Dairy-Met: Compositional metagenomic analysis of milk and cheeses

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By

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This thesis is dedicated to the loving memory of ***Amelia***, our little angel.

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of PhD, is entirely my own work and has not been submitted for another degree, either at University College Cork or elsewhere.

Signed: Lisa Quigley

Date: 4th October 2013

Thesis Abstract

Here we have applied next-generation DNA sequencing technologies to gain a detailed understanding of the microbial content of milk and cheeses. To begin, it was necessary to employ a DNA extraction protocol that could be applied to milk and cheese matrices. Seven methods, including five commercial kits and two traditional phenol-chloroform extraction methods, were assessed. It was determined that a commercial kit, the PowerFood^{TM} Microbial DNA Isolation kit (MoBio Laboratories Inc.), most effectively provided DNA of high yield and purity for downstream applications, including PCR and real-time quantitative PCR (qPCR). Further amendments of the associated protocol highlighted the benefits of including an additional heat treatment step.

Having developed the aforementioned method, an in-depth assessment of the microbial content of Irish artisanal cheeses, Irish commercial milk pre- and post-pasteurisation, as well as of commercial cheese displaying a pink discolouration defect, was undertaken. Our investigations revealed that Irish Farmhouse cheeses, including 18 soft cheeses, 31 semi-hard cheeses and 13 hard cheeses as well as cheese rinds from 11 of these, contain a highly diverse bacterial population. While we detected many typical cheese bacteria, we also revealed the presence of several genera not typically associated with cheese including *Faecalibacterium, Prevotella* and *Helcococcus*. The presence of *Arthrobacter* and *Brachybacterium* in goat's milk cheese was also reported for the first time.

The application of a series of culture-independent methods, including flow cytometry, qPCR and next-generation DNA sequencing, to evaluate the bacterial load of milk pre- and post- pasteurisation, provided a number of interesting insights. Flow cytometry was performed to divide the bacterial populations of raw and pasteurised milk into those which were live or dead. To determine the composition of live bacteria in these milks, we applied DNA sequencing and incorporated a live/dead bacterial stain, ethidium monoazide (EMA), to allow only living or metabolically active bacteria to be detected. The results again revealed the presence of a diverse bacterial population, including a number of genera, *Bacteroides,*

Faecalibacterium, Prevotella and *Catenibacterium*, not previously associated with the microbiology of milk. This analysis also reveals the potential for microbes, usually thought to be eliminated by pasteurisation, to survive commercial pasteurisation, albeit most likely in a stressed or 'viable but non-culturable' (VBNC) state.

There have been reports of pink discolouration defects in commercially produced cheeses for many decades. This problem is sporadic, global and the associated cause has not been determined to date. We examined three types of cheeses in which the problem is encountered, including Swiss-type cheese, "thermophilic"-Cheddar type cheese and Cheddar cheese with coloured annatto. Sequencing data revealed the presence of greater proportions of DNA corresponding to the genus *Thermus* in defective Swiss-type and "thermophilic"-Cheddar type cheeses. In contrast, the microbial composition of the control and defect Cheddar cheese with coloured annatto did not differ; this phenomenon is most likely due to physicochemical factors in this cheese type. Further PCR and DNA sequencing resulted in the more accurate identification of the pinking defect-associated bacterium as *Thermus thermophilus*. Following the production of three experimental Swiss-type cheeses spiked with *T. thermophilus*, and of control cheeses, it became apparent that the pink discolouration occurred in the spiked cheeses only. This pinking was significantly greater when the levels of the starter cultures were adjusted i.e. an increase in *Lactobacillus helveticus* with or without a decrease in *Streptococcus thermophilus*. Monitoring of the dairy processing environment revealed the presence of *T. thermophilus* at multiple locations, with hot water representing the most likely source of cheese contamination.

Finally, next-generation Illumina sequencing was employed to identify single nucleotide polymorphisms in the genome of three strains related to the dairy strain *Lactobacillus helveticus* DPC4571. These strains included two derivatives of DPC4571, which have previously been found to differ with respect to the number of IS elements present, i.e. one contained 18 ISL2 elements (ISL2+) while the other had 15 ISL2

elements (ISL2-), thus contrasting with the 17 ISL2 elements in DPC4571. The third strain investigated was *L. helveticus* DPC5607, a close relative of DPC4571. Phenotypic differences between these three respective strains and with DPC4571 were noted. Genome comparisons allowed us to identify the presence of a number of SNPs occurring throughout these genomes, including SNPs in protein-coding genes involved in essential biological functions, such as transport of cellular components, cell structure and function as well as enzymes likely to be involved in important flavour pathways during cheese ripening. A number of other SNPs were located in hypothetical proteins and mobile elements as well as in intergenic regions, which may also impact on the function of nearby genes. The SNPs occurring in sugar transport genes, oligopeptide transport and purine biosynthesis may explain observed growth differences between the strains.

These studies have revealed the significant merits of employing next-generation DNA sequencing to study microbes of dairy foods. The ability of this technology to reveal the presence of bacteria not previously associated with milk or cheese, including revelations with respect the microbial cause of a cheese defect first reported over 60 years ago, are particularly noteworthy.

Publications

Quigley L, O'Sullivan O, Beresford TP, Ross RP, Fitzgerald GF & Cotter PD (2011) Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese. *International Journal of Food Microbiology* **150**: 81-94.

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Quigley L, O'Sullivan O, Stanton C, Beresford TP, Ross RP, Fitzgerald GF & Cotter PD (2013) The complex microbiota of raw milk. *FEMS Microbiology Reviews* **37**: 664-698.

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Glossary of Terms

°C	Degrees Celsius
ACE	Angio-tension converting enzyme
BLAST	Basic Local Alignment Search Tool
BWA	Burrows Wheeler
cfu	Colony forming unit
CIP	Clean-in-process
CNS	Coagulase-negative staphylococci
CO2	Carbon dioxide
DGGE	Denaturing gradient gel electrophoresis
DHPLC	Denaturing high performance liquid chromatography
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
dsH2O	Distilled water
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EMA	Ethidium Monoazide
EPS	Exopolysaccharide
EU	European Union
FAA	Free amino acid
FAO	Food and Agricultural Organisation
FCM	Flow cytometry
FISH	Fluorescent in-situ hybridization
FITC	Fluoresein Isothiocyanate
FSC	Forward light scatter
FWCH	Fluorescent whole cell hybridization
g	G-force
g	Weight in grams
GC	Guanine-cytosine content
GM17	M17 media with glucose
GRAS	Generally recognised as safe
H_2O	Water
HeNe	Helium Neon
HTST	High temperature short time
IS	Insertion sequence
iSNP	Intergenic SNP
ITS	Intergenic transcribed spacer
L	Litre
LAB	Lactic Acid Bacteria
LB	Luria broth
LH-PCR	Length heterogenity-PCR
MEGAN	MEtaGenome Analyzer
mg	Milligram
MID	Molecular identifier
min(s)	Minute(s)
MiXS-MIMARKS	Minimum information about a marker gene sequence (MIMARKS) and minimum information about any (x) sequence (MIxS) specifications
ml	Millilitre
mMRS	Modified MRS
mW	Mega watts
NaOH	Sodium hydroxide
NCBI	National Centre for Biotechnology Information
ng	nanogram
nm	Nanomole
NSLAB	Non-starter lactic acid bacteria
nsSNP	Non-synonymous SNP
nt	Nucleotide
NWS	Natural whey starter

OTU	Operational taxonomic unit
p	Statistical p-value
PCoA	Principal Co-ordinate Analysis
PCR	Polymerase Chain Reaction
PDO	Protected Designation of Origin
PFGE	Pulse field gel electrophoresis
PI	Propidium iodide
QIIME	Quantitative Insighted Into Microbial Ecology
qPCR	Quantitative real-time PCR
RAPD	Random amplified polymorphic DNA
RDP	Ribosomal Database Project
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal RNA
RSM	Reconstituted skim milk
RT-PCR	Reverse transciption-PCR
SN	Soluble nitrogen
SNP	Single nucleotide polymorphism
SSC	Sideward light scatter
SSCP	Single stranded conformation polymorphism
STEC	Shiga-toxin producing Escherichia coli
TE	Tris EDTA
TKT	Tierra Kavanaugh Turner media
Tm	Melting temperature
TO	Thiazole orange
T-RFLP	Terminal restriction fragment length polymorphism
tRNA	Transfer RNA
TTGE	Temporal temperature gradient gel electrophoresis
v/v	Volume/volume
VBNC	Viable but non culturable
w/v	Weight/volume
WHO	World Health Organisation
µg	Microgram
µl	Microlitre

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Literature Review I

Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese

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Abstract

The availability and application of culture-independent tools that enable a detailed investigation of the microbiota and microbial biodiversity of food systems has had a major impact on food microbiology. This review focuses on the application of DNA-based technologies, such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE), single stranded conformation polymorphisms (SSCP), the polymerase chain reaction (PCR) and others, to investigate the diversity, dynamics and identity of microbes in dairy products from raw milk. Here, we will highlight the benefits associated with culture-independent methods which include enhanced sensitivity, rapidity and the detection of microorganisms not previously associated with such products.

1. Introduction

Raw milk is known to harbour a complex microbial community. Indeed the high nutritional value of this food, its high water content and near neutral pH allows the growth of many microbes (Frank, 1997). These microorganisms include bacteria of technological relevance such as the lactic acid bacteria (LAB), which can contribute to subsequent desirable fermentative reactions (Fox, 1999). However, the presence of spoilage bacteria can have considerable negative effects on the quality of milk and dairy products (Cousin, 1982) while the presence of pathogens can have more severe repercussions. The traditional means of determining the nature of the microbiota present in milk is culture based. The culturable microbiota of milk consists primarily of LAB such as *Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Weisella* and *Pediococcus*. While strains of other genera such as *Propionibacterium*, *Staphylococcus*, *Corynebacterium*, *Brevibacterium* also occur, yeasts and moulds may also be present (Coppola et al., 2008), there also exists a large spectrum of other microbes which occur less frequently or are more difficult to detect. Culture-based methods rely on the isolation and cultivation of microorganisms prior to their identification on the basis of phenotype or genotype. However, it has become apparent that approaches that include a culturing step can lead to inaccuracies due to species present in low numbers being out-competed in laboratory media by numerically more abundant microbial species (Hugenholtz et al., 1998) or the fact that others may simply not be amenable to cultivation in the laboratory (Head et al., 1998). For these reasons approaches to assess the microbial composition of food have had to change dramatically. To address this, there has been an increased focus in recent years on the use of culture-independent investigations through the direct analysis of DNA (or RNA) from food without a culturing step (Table 1; Figure 1) (Coppola et al., 2008, Jany and Barbier, 2008). These represent rapid, sound, reliable and effective methods for the detection and identification of the microorganisms present in dairy products.

2. Culture-independent analysis

Culture-independent approaches have been used increasingly to determine the composition of complex microbial communities. These procedures have enabled the simultaneous characterisation of whole ecosystems and the identification of many species from these sources. The shift from culture-dependent assessment to culture-independent analysis has led to a revolution in microbial ecology. These techniques provide a more sensitive and rapid method than conventional culture-dependent analysis with the major benefit of detecting microorganisms which are difficult to culture or uncultivable. Unfortunately, while such approaches possess numerous advantages over culture-based methods, there can still be some limitations. A number of key factors to consider when employing a culture-independent approach are described below.

2.1 Differentiating between the DNA of live and dead microorganisms

Culture-independent assessment most frequently relies on the analysis of nucleic acids isolated from an entire microbial population. DNA is the focus of analysis in the majority of such studies and provides information with respect to the bacterial diversity and overall microbial history of the environment in question. However, the analysis of DNA does not typically enable one to distinguish that from living and dead cells. In recent years there has been an increased use of DNA stains, such as ethidium monoazide (EMA) (Rudi et al., 2005) or propidium monoazide (PMA) (Josefsen et al., 2010), which penetrate and stain the DNA of dead cells and prevent the subsequent PCR amplification thereof. Alternatively, RNA can be used as a live-cell specific target which also allows one to monitor the active microflora (Duthoit et al., 2005, Rantsiou et al., 2008a, Alessandria et al., 2010, Rantsiou et al., 2008b). While RNA-based studies frequently provide the same results as their DNA-based counterparts, they can on occasion facilitate the identification of microbes not detected by DNA (Alessandria et al., 2010). Thus RNA-based analysis can provide a greater understanding of microbial community structure and functionality (Bodrossy et al., 2006).

2.2 Nucleic Acid Extraction

In the case of culture-independent approaches, the outcome is dependent on the extraction of DNA (or RNA) which is representative of the total microbial population and is of sufficiently high concentration and purity. This can be problematic as the presence of natural compounds such as fats, carbohydrates, proteins and salts can hamper nucleic acid extraction as well as downstream application (Wilson, 1997). However, nucleic acid extraction can be improved by the inclusion of various steps such as the mechanical or enzymatic lysis of cells (Lafarge et al., 2004, Parayre et al., 2007), protein digestion (Parayre et al., 2007) and DNA precipitation (Duthoit et al., 2003). More recently the application of commercially available DNA extraction kits has yielded highly pure DNA (Kuang et al., 2009) while reducing laboratory time and removing the need for harmful chemicals.

2.3 Target region

Following the extraction of nucleic acids from the food matrix, the majority of investigations to date have relied on the use of PCR to amplify the region of interest (Figure 1). The most commonly employed targets for identifying species are the 16S and 26S ribosomal RNA (rRNA)-encoding genes, or regions thereof, for bacterial and eukaryote identification, respectively (Cocolin et al., 2002, Florez and Mayo, 2006). These are specifically targeted as a consequence of possessing both highly conserved and highly variable domains. The existence of conserved regions facilitates the use of universal PCR primers to amplify portions of the gene while analysis of the hypervariable regions allows the identification of the corresponding microorganisms (Delbes and Montel, 2005). In situations where only a region of the 16S rRNA gene is targeted, the V3 region is most commonly amplified (Callon et al., 2007, Delcenserie et al., 2007, Ogier et al., 2002, Randazzo et al., 2010). However, some authors have suggested that other regions can provide a more in-depth assessment (Aponte et al., 2008, Randazzo et al., 2006). Alternatively, one can target other genes such as those encoding the phenylalanine tRNA synthase (*phe*S) (Zago et al., 2009) or the RNA polymerase B subunit (*rpoB*) (Martin-Platero et al., 2009). It should also be noted that while biases, such as the introduction of heteroduplexes

(Kanagawa, 2003) or chimeric amplicons (Wang and Wang, 1997), may occur during subsequent PCR reactions, these can be minimized through the use of high-quality primers, high-fidelity polymerases and by modifying PCR conditions (Ogier et al., 2002).

2.4 Choice of culture-independent technique

A variety of different methods, such as DNA sequencing, denaturing gradient and/or temporal temperature gradient gel electrophoresis (DGGE/TTGE) or single stranded conformation polymorphisms (SSCP) are employed to differentiate between 16S/26S amplicons with different signature sequences. In-depth details of the principal for each of these techniques have been reviewed recently (Coppola et al., 2008, Jany and Barbier, 2008, Juste et al., 2008, Pogacic et al., 2010, Trmcic et al., 2008, Randazzo et al., 2009a) and a summary of these can be found in Table 1. When choosing a technique, the question being asked must be considered. These technologies may be employed to study the general microbial diversity of an ecosystem (Bonetta et al., 2008, Callon et al., 2007, Duthoit et al., 2003), to identify specific microorganisms present (Delbes and Montel, 2005) or both (Martin-Platero et al., 2009). They may also be used to assess microbes in a semi-quantitative, e.g. DGGE or T-RFLP (Terminal Restriction Fragment Length Polymorphism) (Randazzo et al., 2010, Sanchez et al., 2006), or quantitative, e.g. qPCR (quantitative real-time PCR) (Rasolofo et al., 2010), manner. Alternatively, where an assessment of the distribution of microorganisms in a food matrix is required, techniques such as fluorescent in-situ hybridization (FISH) may be utilized (Ercolini et al., 2003).

Although quantitative methods have many benefits, which, in addition to the ability to quantify, include enhanced precision and specificity, there can be disadvantages. QPCR cannot effectively, simultaneously quantify very large numbers of different targets in a single sample and therefore selection of target genes and the development of specific primers and probes is vital (Juste et al., 2008). Furthermore, in many instances poor detection and a lack of reproducibility can be problems in situations where cell numbers are low and it is thus critical that stringent detection methods are designed (Rantsiou et al., 2008a).

It should be noted that approaches that rely on electrophoretic patterns can, on occasion, suffer from resolution-related issues. These problems can be reduced by, for example, the addition of a GC-clamp to one of the primers to increase resolution when using DGGE (Sheffield et al., 1989). However, problems can persist if the melting behaviour of 16S fragments are highly similar/identical while there can also be a concern that multiple band display due to multiple rRNA copy numbers will result in diversity being overestimated (Ercolini, 2004). These problems may be overcome through careful primer design and the application of different sets of primers has also been shown to reduce these occurrences (Duthoit et al., 2003). A benefit of the use of electrophoretic methods has been that it has provided the possibility to subsequently excise bands from gels, facilitating DNA sequencing and the identification of microbes through comparisons with specific reference strains (Parayre et al., 2007), or, more routinely, using public databases such as GenBank or the Ribosomal Database Project (RDP) (Giannino et al., 2009, Ogier et al., 2004). To date, the use of high-throughput DNA sequencing to directly sequence multiple 16S amplicons simultaneously has not been extensively employed to investigate the diversity of milk and cheese.

3. Application of culture-independent methods to study the microbiota and diversity of milk and cheese

3.1 Evaluation of the microbial diversity of milk

One of the most detailed culture-independent studies of a dairy related food was by Callon et al. (2007), who examined the microbial diversity of goat's milk samples throughout one lactation year. This study relied on SSCP analysis of DNA extracted directly from milk and from isolates, as well as RFLP (Restriction Fragment Length Polymorphisms) typing of isolates. The combined use of these techniques, as well as culturing on a wide selection of media, revealed the presence of a diverse population of bacteria and yeast in the milk (Table 2). In addition to species commonly encountered in milk, some species which are atypical of goat's milk or had previously only been associated with cheeses, including a number of corynebacteria and brachybacteria, were identified thereby highlighting the sensitivity of this

approach. Another unexpected finding was the detection of several halophilic species atypical of milk, including *Jeotgalicoccus psychrophilus*, *Salinicoccus* sp., *Dietzia maris*, *Exiguobacterium*, *Ornithinicoccus* sp. and *Hahella chejuensis*, these were detectable by RFLP and SSCP of isolates and from milk DNA extracts. While the two techniques employed each identified many of the same species, the culture based approach was considerably more labour intensive, involving the use of 9 different media, isolate selection, purification and storage. In contrast, SSCP facilitated the rapid detection of the same species (Callon et al., 2007). The analysis carried out also highlighted a seasonal variation in the microbial composition of the milk. This is an important factor when considering the ultimate use of the milk as, for example, some variations may affect the flavour development of cheese (Randazzo et al., 2010). Another factor which has been found to influence the microbial composition of milk is the location of the animal. More specifically, Bonizzi et al. (2009) employed intergenic transcribed spacer analysis (ITS; Table 1) to investigate the composition of milk sampled at different regions, i.e. alpine pasture, valley and lowland farms, of the North-western Italian Alps over a 2-year period. While this approach did not reveal the identity of the species or strains present, evident differences in band patterns between lowland plains, valleys and alpine pastures were apparent. When analysed yearly, cluster analysis indicated that the milk from alpine pastures formed a large, nearly homogenous cluster, while that from valleys showed a tendency to scatter into a number of small mixed groups with some adjoining the alpine pasture cluster. It was noted, however, that some bands were common across the samples suggesting that a number of species were widespread (Bonizzi et al., 2009). This technique was also employed to establish that farm location impacts on the microbial composition of cheese. This is an important consideration with respect to the discrimination of cheeses especially in the context of Protected Designation of Origin (PDO) cheese (Bonizzi et al., 2007). A more recent study has also highlighted the impact of a cow's feeding environment on milk microbial composition. Here, DGGE and qPCR were employed to reveal that the predominant species, regardless of whether feeding occurred indoor and outdoor, were lactobacilli, but that *Staphylococcus* species appeared in milk after 8 days of outdoor feeding only

(Hagi et al., 2010). Finally, it is also important to consider the yeast population of milk, which can impart important flavours on dairy products (De Freitas et al., 2009). Cocolin et al. (2002) carried out such analyses which focused on the yeast population in raw cow's milk and used a combination of culturing and DGGE fingerprinting. The classical culture-based methods identified six *Candida* species (which accounted for 56.2% of the population), two *Kluyveromyces* species, *Saccharomyces cerevisiae*, *Pichia guilliermondii*, *Trichosporon mucoides* and *Cryptococcus curvatus* as being the main yeast constituents. When the samples were analysed by DGGE additional components such as *Galactomyces* sp., *Candida kefyr*, *Candida humilis* and *Saccharomyces bayanus/pastorianus* were detected (Table 2) (Cocolin et al., 2002).

3.2 Evaluation of the microbial diversity of whey starters

While the manufacture of cheese frequently can involve fermentation by the natural microbial population only, i.e. no starter culture addition, there are many cheeses which use starter cultures. These may be defined starters, a known culture or a mix of known cultures which contribute to specific cheese traits during manufacture or in some instances natural whey starters (NWS). NWS have a complex microbial association of various species as well as a large number of biotypes (Giraffa et al., 1997). The complex microbiota of these whey starters has been investigated in recent years using culture-independent approaches. In one instance, Length Heterogeneity (LH)-PCR was employed to reveal the dominance of *Lactobacillus helveticus* and *Lactobacillus delbrueckii* and, to a lesser extent, *Streptococcus thermophilus* and *Lactobacillus fermentum* (Santarelli et al., 2008). In another instance, the investigation of whey starters was facilitated by reverse-transcription (RT)-PCR amplification of the 16S rRNA transcript (i.e. assessing viable cells only) followed by culture-independent T-RFLP analysis, to monitor the population dynamics of the metabolically active fraction in the microbiota of a defined starter over a fermentation period (Sanchez et al., 2006). In this instance T-RFLP was used as a semi-quantitative means of analysis (on the basis of peak area ratios). Ultimately, some differences between the data generated by T-RFLP and colony counting were noted when monitoring *Lactococcus*

lactis subsp. *lactis* and *Leuconostoc citreum*. Although, over the first 9 hours, both approaches yielded similar results, after 24 hours there was a clear difference as T-RFLP detected higher levels of *L. lactis* subsp. *lactis* than were revealed by colony counting, i.e. approximately 90% compared to 80%, respectively. This indicates that a proportion of these cultures remained metabolically active but in a viable but non-cultivable (VNC) state and thus were not detectable by culture-dependent methods (Sanchez et al., 2006). Finally, the bacterial diversity of natural whey cultures has also been investigated using denaturing high performance liquid chromatography (DHPLC) as well as DGGE, with results comparable to those noted previously in that *L. delbrueckii*, *L. helveticus*, *S. thermophilus* and *L. lactis* were all detected (Ercolini et al., 2008).

3.3 Evaluation of the microbial diversity of different cheeses

There have been a considerable number of molecular-based studies dedicated to investigating the microbial composition of cheese. Here we divide these investigations into different subcategories i.e. (3.3) a comparison of the microbiota of different cheeses, (3.4) a comparison of the outcomes when these investigations are carried out using culture-dependent and -independent approaches, (3.5) investigations focusing on population dynamics during the fermentation process as well as (3.6) studies that focus on the detection of spoilage related and pathogenic microorganisms.

First we will summarise investigations which have highlighted the large diversity of the cheese-associated microbes, which is itself a reflection of the great diversity in cheese making approaches. The most consistent observation across these studies is the increased microbial diversity apparent in artisanal, relative to industrially manufactured cheeses, regardless of the type of cheese. This pattern undoubtedly reflects the frequent use of raw milk and undefined starters in artisanal cheeses. Four types of mozzarella cheeses were the focus of attention when DGGE was first employed to investigate a dairy microbial environment (Coppola et al., 2001). These 4 cheeses were made from (a) pasteurised cow's milk and commercial starters, (b) raw water-buffalo milk and natural whey cultures, (c) raw cow's milk and natural thermophilic milk cultures and (d) raw cow's milk without a starter culture,

respectively. The analysis of cheeses (a) and (c) led to the detection of *S. thermophilus* only whereas *S. thermophilus*, *L. lactis* and *Lactobacillus* species were detected in cheese (b) and (d). Cheese (d) also contained *Enterococcus faecalis* and *Leuconostoc lactis*, thereby highlighting that the greatest diversity resulted from the use of raw milk and the absence of starter (Coppola et al., 2001). Similar such studies, which again employed a DGGE approach, but which focussed on cheese produced from ewe's or goat's milk, have also revealed the presence of a more diverse flora in artisanal cheeses (Randazzo et al., 2006, Bonetta et al., 2008) (Table 3). In another instance, the focus turned to the use of SSCP, as well as culturing on brain heart infusion media, to assess the impact of pasteurisation on rind development on red-smear soft cheeses (Feurer et al., 2004). This approach revealed bacteria which were common to both cheeses (Table 2), but also highlighted the specific association of *Carnobacterium maltaromaticum*, *L. lactis* subsp. *cremoris, Sporanaerobacter acetigenes* and an uncultured Proteobacteria with the pasteurised milk cheese smear whereas the raw milk cheese smear exclusively contained *Corynebacterium casei, Lactobacillus curvatus* subsp. *curvatus, Marinilactibacillus psychrotolerans, Microbacterium gubbeenense*, *Brachybacterium*, *Lactobacillus sakei, Pseudoalteromonas* species, and an uncultured *Flavobacteriaceae* within its surface smear (Feurer et al., 2004). Additional observations made by other groups include the examination of four different commercial cheeses (two produced from raw milk and two from pasteurised milk) again revealing a greater diversity of the microbes in the raw milk cheeses (Ogier et al., 2004). This study was also notable as it recorded, for the first time, the presence of *Pseudoalteromonas* and *Halomonas* in a cheese core (Ogier et al., 2004). The ripening conditions employed also have a significant impact on the microbial composition of cheese. This fact was revealed when TTGE fingerprinting and RAPD (Random Amplified Polymorphic DNA) analysis was used to compare the microbiota of two farmhouse cheeses manufactured from raw goat's milk in the absence of a starter culture. The key difference related to the fact that the cheeses were ripened as hard (Quesailla Arochena) and soft cheeses (Torta Arochena), respectively (Martin-Platero et al., 2009). Although some species were common to both cheeses, only the hard cheese contained *Hafnia alvei, Leuc.*

lactis and *Mycobacterium agalactiae* while *Serratia liquefaciens, Leucobacter* species and *E. faecalis* were detected in the soft cheese exclusively (Martin-Platero et al., 2009). It is also worth noting that a study comparing the microbial composition of raw and pasteurised milk revealed higher levels of *Enterobacteriaceae, Citrobacter* sp. and *Bacillus* species in pasteurised milks whereas raw milk was dominated by *Bifidobacterium* and also contained more *L. lactis*, *S. thermophilus*, *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Corynebacterium afermentans* (Duthoit et al., 2005).

Other culture-independent techniques have also been employed to investigate the microbial composition of a large variety of cheeses. These include techniques such as T-RFLP, which detected staphylococci, microbacteria, brevibacteria and corynebacteria on the Tilsit cheese surface. T-RFLP also revealed the presence of *Carnobacterium* which went undetected when culture-dependent methods were employed (Rademaker et al., 2005). Another technique which has been applied with success to study the microbial composition of cheese is FISH. In one instance this method has determined the distribution of LAB in different regions of Stilton cheese. This study revealed that the cheese core was dominated by *L. lactis*, that the veins and surface were dominated by lactobacilli and *Leuconostoc* and that other unidentified coccoid microorganisms were also detected at lower levels throughout (Ercolini et al., 2003). FISH analysis has also been employed to characterise the yeast population of Livarot cheese surface, revealing that *Candida catenulata* and *Geotrichum* species dominate (Mounier et al., 2009). A similar approach, i.e. fluorescent whole cell hybridization (FWCH), detected the presence of *Enterococcus italicus*, a recently described dairy-associated enterococcal species, in raw milk cheese (Fornasari et al., 2008).

3.4 Comparison of outcomes when culture-dependent and culture-independent approaches are used to assess the microbiota of milk and cheese

Culture-independent approaches have a tremendous advantage in that they can reveal microbes that are difficult, or impossible, to culture. While a number of studies, such as that by Van Hoorde et al. (Van Hoorde et al., 2008), have highlighted this benefit, others have benefited from using parallel

culture-dependent and -independent based approaches (Table 2). Indeed, discrepancies with respect to the detection of particular species were noted when different approaches were taken to investigate the microbiota of raw milk Alberquilla cheese i.e. culture-dependent approaches detected *Leuconostoc mesenteroides*, *Leuconostoc pseudomesenteroides* and *Pediococcus urinaequi* while culture-independent TTGE revealed *Lactobacillus acidophilus* and an unidentified nitrogen-fixing bacterium (Abriouel et al., 2008). In a Lebanese artisanal raw goat's milk cheese, *L. curvatus* and *S. thermophilus* were identified by culture dependent assessment but *Staphylococcus haemolyticus*, *Streptococcus* sp., *Escherichia coli*, *Clostridium bifermentans* and *Eubacterium tenue* were detected by culture-independent TTGE only (Serhan et al., 2009). A Camembert cheese was found to contain, on the basis of culture-dependent investigations, *L. delbrueckii* subsp. *bugaricus* and subsp. *lactis* as well as *Lactobacillus casei* subsp. *casei* but culture-independent TTGE instead revealed the presence of *Lactobacillus paracasei* subsp. *paracasei*. With 16S rRNA gene analysis it can be difficult to distinguish between the *L. casei* and *L. paracasei* which may be responsible for these apparent differences (Henri-Dubernet et al., 2004). In Feta cheese, culturing and DGGE analysis of the bacteria present were inconsistent but corresponding analyses of the yeast population provided comparable results (Rantsiou et al., 2008b) (Table 2). Notably, the culture-independent approaches revealed that a number of thermophilic LAB, including *L. plantarum, S. thermophilus* and *Lactococcus* species were in a VBNC (viable but non-culturable) state (Rantsiou et al., 2008b). In raw ewe's milk cheese Pecorino Crotonese, culture-dependent assessment detected the majority of the microbes revealed by culture-independent, TTGE analysis (Table 2), however culture-independent TTGE also revealed a more diverse *Lactobacillus* population which included *Lactobacillus buchneri*, *L. fermentum*, *L. delbrueckii* and *L. plantarum/pentosus* (Randazzo et al., 2009b). It was noted that only culture-independent DGGE succeeded in detecting *Leuc. mesenteroides* in raw goat's milk Bukuljac cheese (Nikolic et al., 2008). Culture-independent DGGE assessment also revealed, for the first time, the presence of *S. thermophilus,* in the Spanish raw cow's milk cheese, Casin (Alegria et al., 2009). In

Parmigiano Reggiano cheese, *Pediococcus acidilactici* was detected by culture–dependent approaches only whereas *L. fermentum* was exclusively identified by culture-independent (DGGE) (Gala et al., 2008), a study of Saint-Nectaire cheese revealed significant differences between culture-dependent and culture-independent (SSCP) results (Table 2) (Delbes et al., 2007) and, finally, in Salers cheese, culture-dependent methods detected a more diverse yeast population consisting of *Candida intermedia*, *Candida tropicalis*, *Candida rugosa* and *P. guilliermondii* (Callon et al., 2007, Callon et al., 2006). Detailed results of these studies can be found in Table 2.

These observations highlight the benefits of applying a polyphasic approach, i.e. culture-dependent and culture-independent, when assessing a microbial community. However, it is worth noting that in many of the studies where culture-dependent methods highlighted a more diverse microbial composition, these analyses also employed molecular techniques (DGGE/TTGE and SSCP) to then identify the isolates (Delbes et al., 2007, Henri-Dubernet et al., 2008), thus, emphasising our increasing reliance of culture-independent technologies to determine microbial diversity.

3.5 Investigation of microbial composition and succession during cheese manufacture

In addition to facilitating an analysis of the microbiota of milk and cheese diversity, as well as the distribution of microorganisms throughout different regions of a cheese, culture-independent fingerprinting has been used to assess how these microbial populations shift from that present in milk at the beginning of the fermentation process, throughout curd maturation, until the final cheese. The first application of SSCP fingerprinting to obtain such an insight focused on the production of Salers cheese from raw cow's milk (Duthoit et al., 2003). In the study the authors detected a highly diverse microbiota, which included a variety of LAB which dominated the microbiota throughout fermentation. The application of SSCP fingerprinting in this study was also notable for the fact that it revealed, for the first time, the presence, of coryneform bacteria, i.e. *Coryneform variabilis, Coryneform afermentans*, *Coryneform bovis* and *Coryneform flavescens*, in a cheese core (Duthoit et al., 2003). Another study, which applied DGGE to profile the microbial pattern

of Cabrales cheese, a Spanish raw cow's milk cheese, revealed that the initial microbiota of the raw milk was remarkably similar to that of the ripened cheeses, with *L. lactis* subsp. *lactis* populations being dominant throughout the process. It was apparent, however, that lactobacilli appeared during ripening. DGGE also detected the presence of low levels of *Bifidobacterium psychroaerophilum* in cheese after 90 days and revealed that the fungal flora changed significantly after day 15 of cheese ripening (Figure 3) (Florez and Mayo, 2006). Notably, the microflora of Cabrales cheese had previously been assessed using conventional plating methods (Florez et al., 2006). Although a comparison of both studies revealed a broadly similar outcome, it was apparent that *B. psychroaerophilum* was detected by culture-independent approaches only and that this strategy highlighted a more diverse *Lactococcus* population. In a similar study, DGGE analysis was applied to monitor microbial diversity during the production of raw milk, Fontina, cheese. The raw milk was dominated by LAB, which is consistent with previous culture-based studies (Senini et al., 1997), but also contained *Staphylococcus* sp., *Pantoea sp., Chryseobacterium* and *Moraxella*. After heating of the milk (47-48°C) to form curds, the bacterial population became more homogenous with *S. thermophilus* and enterococci dominating the fresh curd population. These changes also coincided with the appearance of *Kocuria rhizophila* and *Klebsiella oxytoca*, an increase in *Pantoea* sp. and a decrease in *Chryseobacterium*, *Moraxella*, and *Macrococcus* sp. (Giannino et al., 2009).

While the comparisons referred to in the previous paragraph were possible due to the availability of data from related culture-based studies, other studies have employed traditional culturing approaches in parallel with modern culture-independent assessments to gain further insight into the complex microbial changes that occur during dairy fermentations. Ercolini et al. have monitored the microbiota of whole raw buffalo milk and the changes which occur during the production of traditional water buffalo mozzarella cheese using PCR-DGGE and culturing. The DGGE fingerprint revealed that the milk microbiota changes after starter addition, in the form of a natural whey culture (NWC), and the cheese microbiota remained similar to the NWC from its addition until the final cheese product was generated. The parallel culture-based approach established that buffalo milk is rich in LAB, with an

increase in both thermophilic and mesophilic microorganisms occurring after starter addition (Ercolini et al., 2001, Ercolini et al., 2004). Another such study, employing culture-based and DGGE fingerprinting analysis of a raw cow's milk cheese, detected a diverse LAB population present from raw milk to the final cheese (Randazzo et al., 2002). Furthermore, RNA was extracted from milk and cheese and assessed by RT-PCR-DGGE. Although the results of RNA- and DNA-generated patterns were comparable, differences existed in that the RNA-derived results showed that *L. lactis* and *Leuc. mesenteroides* were not as metabolically active in milk and curd as had been anticipated whereas *L. acidophilus* and *L. delbreuckii* subsp. *bulgaricus* were particularly active (Randazzo et al., 2002).

A raw cow's milk cheese, Castelmagno, which was manufactured without the addition of starter cultures during summer, was also assessed by both DGGE and culturing methods, again revealing a good correlation between culture-dependent and -independent methods (Dolci et al., 2008). DGGE revealed a predominance of *L. lactis* subsp. *lactis* and subsp. *cremoris* throughout both the cheese making and ripening stages. DGGE also more effectively detected *L. plantarum*, *Macrococcus caseolyticus* and *Streptococcus agalactiae* during the early stages and *Lactobacillus kefiranofaciens* from the latter stages of the process (Table 2). This information was supplemented by culture-derived results which established the presence of lactobacilli throughout the cheese making and ripening process (Dolci et al., 2008). Interestingly, Castelmagno that had been produced in winter was subsequently assessed using the same approach but in this instance an RNA-based investigation was also carried out (Dolci et al., 2010). The culture-based approach established that lactococci and lactobacilli increased from milk to the curd up to day 30 of ripening after which time they began to decrease. In contrast, DNA and RNA fingerprinting determined that *L. lactis* dominated, and continued to be metabolically active, until the end of ripening. In addition to these specific outcomes, it is important to note that a seasonal influence on microbial population levels was also apparent (Dolci et al., 2010).

Combinations of conventional and culture-independent (DGGE) approaches were used to study the lactic acid bacteria of raw cow's milk

cheese, Provolone del Monaco (Aponte et al., 2008). All approaches revealed that *S. thermophilus* and *Streptococcus macedonicus*, and to a lesser extent enterococci, dominated throughout the manufacturing process. An increase in lactobacilli populations was detected during ripening, although it was noted that *L. delbreuckii* was detected by DGGE only (Table 2). Interestingly, a comparison of three different DNA extraction protocols highlighted the importance of using a suitable extraction method (Aponte et al., 2008). A Croatian raw sheep's milk cheese has also been assessed by culture dependent and independent methods (Fuka et al., 2010). DGGE analysis revealed a more diverse microbial composition, detecting the presence of *E. faecium/faecalis, L. lactis* subsp*. lactis, Enterobacter cancerogenus, Klebsiella terrigena, Klebsiella pneumoniae, Pantoea agglomerans, Entrobacter hormaechei, Staphylococcus equorum, Staphylococcus sciuri, Staphylococcus gallinarum, Staphylococcus saprophyticus* and non-fermentative *Acinetobacter* in milk with *M. caseolyticus* and *Pseudomonas fragi* being sporadically identified in milk and the fresh curd. After 30, 90 and 120 days ripening, the dominant species was *L. lactis* subsp. *lactis* with the presence of *L. curvatus, S. saprophyticus* and *Enterococcus* species also being noted (Fuka et al., 2010). Alessandria et al. (2010) evaluated the dominant population of an artisanal cheese produced on the Cape Verde Islands. Here, the authors employed culture-dependent methods, alongside DNA and RNA culture-independent PCR-DGGE methods. Overall, the culturing and DGGE profiling revealed similar results (Table 2). However, a number of bacteria were only detected by the RNA-based strategy. These included *Moraxella oslonesis, L. helveticus, Leuc. pseudomesenteroides* and *K. rhizophila* (Alessandria et al., 2010). Finally, the use of DGGE by Randazzo et al. (2010) has highlighted the diversity of the LAB population in Pecorino Crotonese cheese. *L. lactis* subsp. *lactis* dominated throughout the process with *Lactobacillus brevis* and *L. buchneri* also being detected. During ripening, a number of other species, including *L. plantarum/pentosus*, *L. fermentum, Leuc. mesenteroides*, *L. delbrueckii* and *Lactobacillus rhamnosus*, were identified and it was revealed that *S. thermophilus* became dominant at this point. On the basis of the appearance and disappearance of bands, the authors were able to assess the impact of the microbial composition on

flavour development. It was apparent that when LAB species were abundant in the final product, there were higher concentrations of volatile compounds which contribute floral and fruity notes (Randazzo et al., 2010).

3.6 Application of molecular biology for pathogen and spoilage related investigations of milk and cheese products

DNA-based technologies benefit from being capable of providing a rapid assessment of the composition of a microbial niche. This is particularly important when determining the presence of pathogenic microbes in dairy products. Quantitative PCR has been particularly beneficial here allowing the rapid identification and quantification of such microorganisms. A number of studies have developed and validated qPCR methods to monitor pathogens such as *Mycobacterium avium* subsp. *paratuberculosis* (MAP), *Staphylococcus aureus* and *Listeria monocytogenes* (Slana et al., 2008, Graber et al., 2007, Rantsiou et al., 2008a). MAP, the causative agent of Johnes disease in cow's and which has, on occasion, been associated with Crohn's disease in humans (Ayele et al., 2001), is a problem for the dairy industry. Because of concern and debate regarding the possibility that milk may serve as a vehicle for the transmission of MAP to humans, the rapid detection of MAP in milk and dairy products is of key importance to dairy microbiologists (Hermon-Taylor and Bull, 2002). Culturing of this microbe is long and laborious, often taking months and with no guarantee of success. In a study of 345 milk samples, the culture-dependent approach, which was conducted over 8 months, failed to detect MAP, however 111 of the samples were found to be positive when an alternative, rapid qPCR-based approach was taken (Slana et al., 2008). Similar patterns have been observed in subsequent studies of milk and cheese (Slana et al., 2009, Botsaris et al., 2010). In each case qPCR was capable of detecting MAP at levels below 10 cells per ml of milk. While these studies highlight the importance of a culture-independent approach, it should also be noted that the detection of MAP by these methods were DNA-based and that a RNA approach could also be conducted to reveal if the MAP in question are dead DNA or in a viable but non-cultivable state.

Another microorganism of interest to the dairy industry is *S. aureus*, the primary cause of mastitis infection in cows and a significant food pathogen. Graber et al. (2007) devised a qPCR method which facilitated the rapid detection of *S. aureus* in bovine milk at low concentrations (1-10 cfu ml^{-1}; i.e. 50 times more sensitive than plating). The benefits of employing qPCR to detect *S. aureus* in milk have also been highlighted in other studies (Studer et al., 2008). While a broad variety of target specific DNA-based approaches, including qPCR, are available to detect the presence of pathogens, a community-based analysis can also be revealing. Delbès and Montel (Delbes and Montel, 2005) used SSCP analysis in order to detect and discriminate between individual components of the staphylococcal population of a raw cow's milk cheese. This analysis detected the presence *S. aureus*, *S. equorum* and *S. saprophyticus* and revealed the extent to which levels of these species varied during the cheese-making process, with major increases in *S. aureus* from raw milk to the 12 hour cheese being particularly notable (Delbes and Montel, 2005). Similarly, although the analysis of raw milk produced by four mastitic cow's, by PCR-DGGE revealed the presence of a number of pathogens known to cause infection, i.e. *Escherichia* sp., *Enterobacter* sp., *S. aureus* and *Streptococcus uberis*, the corresponding use of three types of selective agar (Blood, TKT and MacConkey agar) incorrectly indicated that coliforms were the sole cause of infection (Kuang et al., 2009). The accurate identification of the aetiological agent is obviously of key importance with respect to the treatment of mastitis and, thus, in this instance, the risks associated with relying solely on culture-generated information are apparent.

Another pathogen which is of major concern for the dairy industry is *L. monocytogenes*, with soft, raw milk cheeses being particularly problematic. Thus, rapid and accurate detection of this pathogen is critical. Rantsiou et al. (2008a) devised a *L. monocytogenes*-specific qPCR method which was employed to test 33 fresh cheese and 11 ripened cheese samples made from raw goat's milk. This approach revealed that 4 fresh cheese samples were positive for *L. monocytogenes*, a number which increased to 8 after the inclusion of an enrichment step (Rantsiou et al., 2008a). SSCP fingerprinting of raw milk cheese has also been employed to provide an insight into the

microbes that may inhibit *L. monocytogenes* in cheese (Saubusse et al., 2007). Two cheese groups were examined with group I containing high numbers of *L. monocytogenes* whereas the counts in cheeses from group II were low. SSCP analysis of the microbial populations revealed that group II had a greater number of peaks corresponding to *Enterococcus faecium/saccharominimus*, *Chryseobacterium* sp./*flavescens* and *Lactococcus gravieae/lactis* compared to group I. To determine if these species were influencing the presence of *Listeria* in these cheeses, a pasteurised milk cheese model, into which *L. monocytogenes* and the putative inhibiting strains were introduced, was manufactured. Following testing, it was determined that inhibition occurred when *L. lactis* and *L. garvieae*, and to a lesser extent *E. saccharominimus* and *C. flavescens*, were included. Strangely, neither the production of inhibitory compounds, such as bacteriocins, nor a drop in pH seemed responsible, and thus the mechanism via which the pathogen was inhibited was not apparent (Saubusse et al., 2007). This study reveals how culture-independent methods can be applied, to a complex microbial community, to reveal the presence of microorganisms with possible antimicrobial properties.

Clostridia, representatives of which can cause the defect late blowing in cheese, are responsible for massive financial losses in the dairy industry. Detection of these spoilage microorganisms is thus extremely important. In one instance, seventeen raw milk cheeses were subjected to DGGE-based analysis to characterise the clostridial population, leading to the identification of four species i.e. *Clostridium butyricum*, *Clostridium beijerinckii*, *Clostridium sporogenes* and *Clostridium tyrobutyricum* (Cocolin et al., 2004). The clostridial population of twenty raw milk cheeses was the subject of another investigation employing TTGE fingerprinting. Four different species of clostridia, i.e. *C. tyrobutyricum*, *C. beijerinckii*, *C. butyricum* and *C. sporogenes*, were detected by TTGE, which contrasted with the identification of only two species, *C. sporogenes* and *C. tyrobutricum*, when a culture-based approach was employed (Le Bourhis et al., 2005).

SSCP fingerprinting has also been used to monitor how milking practice and farm hygiene influence the microbial composition of milk (Verdier-Metz et al., 2009). Milk samples collected from dairy farms, after

morning milking, were analysed. Milking practices were monitored by surveying the hygiene process applied to udders and the milking system, pre- and post-milking. The samples were divided into three groups on the basis of milking practices and the associated microbiota, as determined by SSCP; Group A being those where hygiene practices were most intensive while Group C was least hygienic. While milk from all three groups contained *L. lactis* at equal concentrations (Figure 4), the samples differed with respect to their overall microbial diversity. This diversity was measured by the Shannon Index and it was revealed that Group A had the lowest diversity (0.88) while Group B (1.09) and Group C (1.18) had higher diversity (Verdier-Metz et al., 2009). Other studies have used these approaches to monitor the impact of storage conditions. Lafarge et al. (Lafarge et al., 2004) revealed that *L. lactis*, followed by *Staphylococcus* species, *K. pneumoniae* and, to a lesser extent, *Listeria, Enterococcus* and *Streptococcus* species, were the major raw milk species present prior to refrigeration. After refrigeration at 4°C for 24 hours, many of the species initially identified were still present but their relative proportions were clearly altered (Table 4). Unsurprisingly, there were increases in the numbers of *Listeria*, which are psychrotrophs (Lafarge et al., 2004). This approach was deemed more sensitive than traditional methods which indicated that incubation for 48 hours at 4°C was required before a bloom in the psychrotrophic microbiota occurred (Brouillaud-Delattre et al., 1997). Culture-independent, real-time qPCR has also been applied successfully to investigate the impact of an even greater variety of factors i.e. thermisation, carbon dioxide (CO_2) and microfiltration at 4°C and 8°C. The investigation revealed that while levels of all species increased when milk was stored at 8^{o}C, *Acinetobacter calcoaceticus, Aerococcus viridans, S. aureus, S. uberis and Corynebacterium* were stable for 7 days at 4°C, regardless of the treatments employed. However, *P. fluorescens* differed in that levels in raw milk and CO_2 treated milk increased over 7 days relative to untreated controls (Rasolofo et al., 2010).

4. Conclusion and future prospects

Since the invention of PCR technology, the field of microbial ecology has evolved with tremendous speed and molecular methods are continuing to revolutionise our understanding of the composition and population dynamics of microbial communities in various environments. The culture-independent methods described in this review have facilitated substantial progress in food microbial ecology by facilitating the simultaneous study of viable, non-cultivable and stressed/injured microbes. Significant advances have included the identification, for the first time, of various microorganisms from milk and cheese, such as coryneform bacteria, *Pseudoalteromonas* and *Halomonas* in the cheese core and *S. thermophilus* in Spanish cheese. Other studies have highlighted the ability of culture-independent methods to rapidly identify microbes of interest to the food industry, including MAP, *S. aureus* and clostridia. While many publications have highlighted the benefits of using polyphasic approaches, i.e. both culture-dependent and culture-independent strategies, it was noted that culture-independent methods were more rapid, sensitive and less susceptible to bias than culture-dependent methods. In addition, the ability to distinguish between dead or viable cells is an important factor in understanding the process of cheese manufacture. Upon lysis, cells release their intracellular components which contribute to cheese development, especially in the context of contributing to the textural, flavour and aroma development in a cheese. However, many cells which appear to be dying on the basis of conventional culturing may be in a permeabilized but viable state and therefore the ability to distinguish between dead and viable cells, using a combination of DNA- and RNA-based approaches, is another important benefit of culture-independent methods. It is thus apparent that culture-independent methods have been of critical importance with respect to our investigation of the microbial community of raw milk and raw milk cheese and our development of a better understanding of the role of these microbes in the flavour, quality and safety of dairy products.

While the techniques describes here have been vital to our understanding of dairy microbiology, the field of microbial ecology is constantly evolving. The last few years have seen the introduction of next-generation sequencing

technologies which are replacing other culture-independent approaches. These technologies provide the benefits of reduced labour time, lower reaction volumes, extended number of sequence reads as well as high-throughput sampling. This has proven extremely successful in profiling the microbiota of various environments including deep sea vents, gut and soil amongst other environments. However, there have been extremely few studies in which these technologies have been utilised to investigate the microbial composition of foods. Those which have taken place have revealed the diverse microbial populations present in fermented seafood (Roh et al., 2010), vegetables (Jeon et al., 2011) and rice bran (Nakayama et al., 2011). Notably, such an approach has also been applied recently to a raw milk cheese (Masoud et al., 2011) revealing a diverse subdominant population which went undetected by DGGE analysis. These studies suggest that the application of next generation DNA sequencing technologies will greatly enhance our research in the area of food microbiology, including that of raw milk and raw milk cheeses.

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Figure 1: Overview of the steps involved in a culture-independent assessment of a food system. This figure highlights the major points to consider for some the main culture-independent methods. Other methods, such as FISH or DHPLC, may involve different processes.

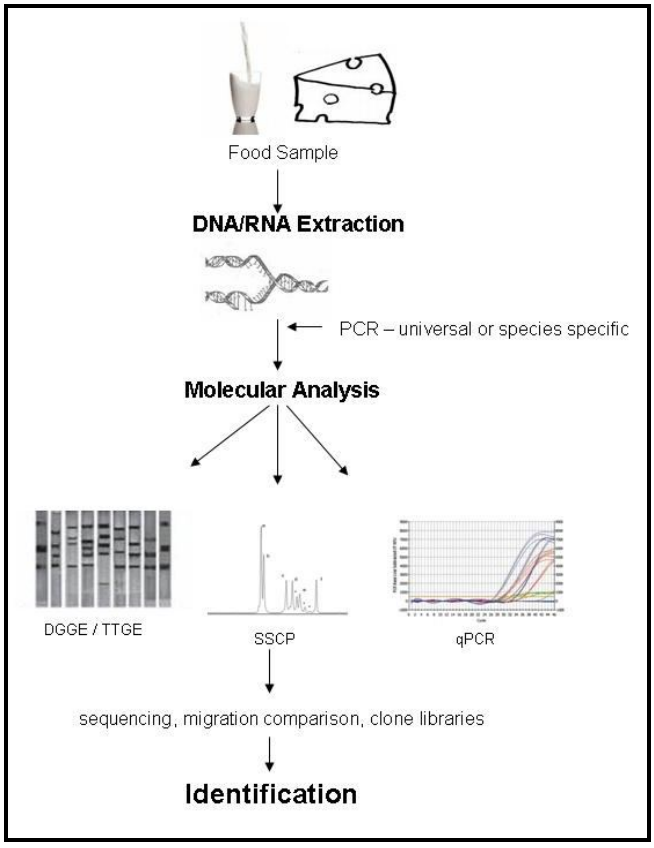


Figure 2: FISH analysis was able to depict the dominant microorganisms in the different areas of stilton cheese, i.e. core, surface and veins (Ercolini et al., 2003).

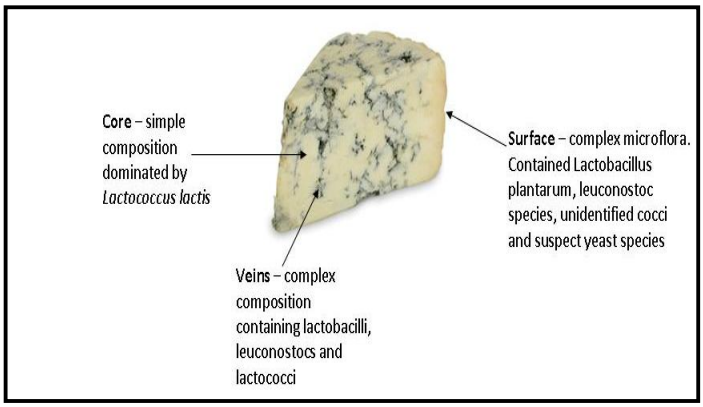
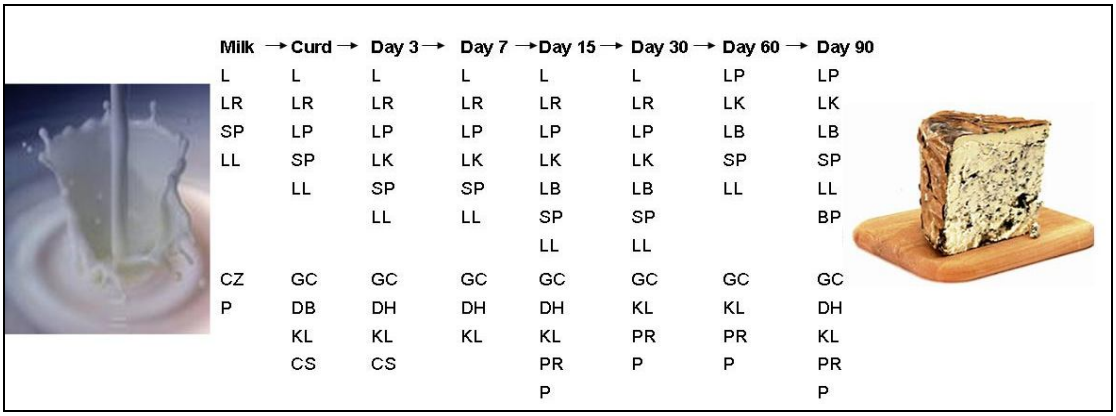


Figure 3: Microbial changes during the manufacture of artisanal Cabrales cheese monitored by DGGE analysis (Florez and Mayo, 2006).



L- *Lactococcus lactis*, LR- *Lactococcus raffinolactis*, SP- *Streptococcus parauberis*, LL- *Lactococcus lactis* subsp. *lactis,* CZ- *Candida zeylanoides*, P- *Penicillium chrysogenum/griseofulvum,* LP- *Lactobacillus plantarum,* GC- *Geotrichum candidum,* DH- *Debaryomyces hansenii,* KL- *Kluyveromyces lactis,* CS- *Candida silvae,* LK- *Lactobacillus kefiri,* LB- *Lactobacillus buchneri,* PR- *Penicillium roqueforti,* BP- *Bifidobacterium psychrophilum.*

Figure 4: SSCP peak assignment. Peaks are assigned based in the percentage of milks in each group where a specific peak is present representing a bacterial species. The percentage is based on the number of milk samples in a group, Group A = 20; Group B = 19; Group C = 28. Developed from results of study conducted by Verdier-Metz et al. (2009).

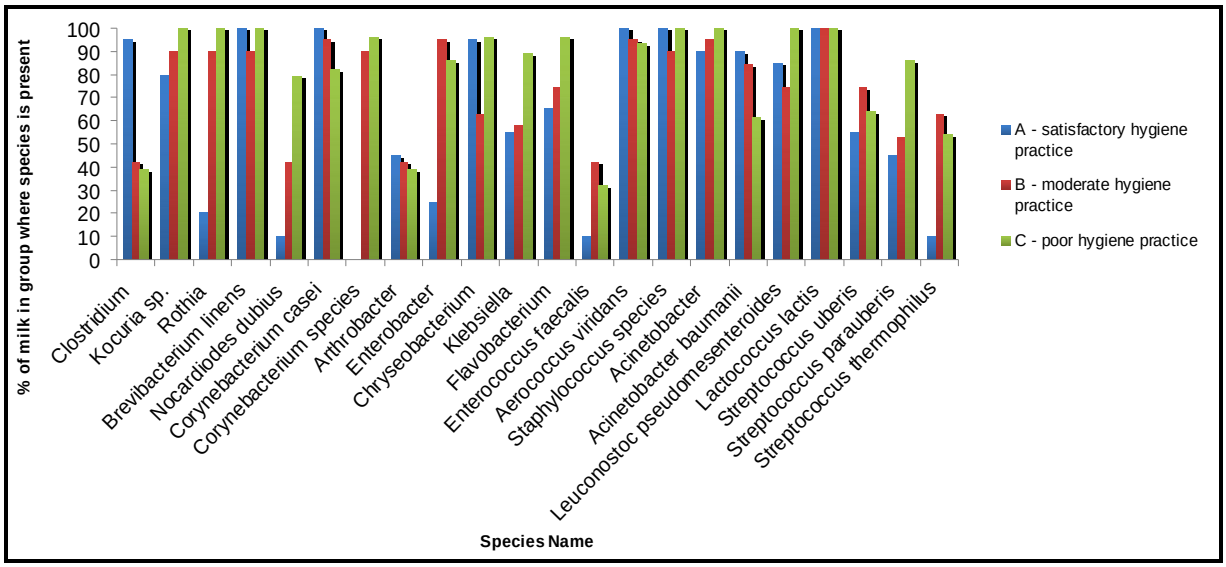


Table 1: Description of the main genomic-based methods involved in describing microorganisms in milk and cheese. Detailed descriptions of these principals have been reviewed recently byJuste et al., 2008, Pogacic et al., 2010, Randazzo et al., 2009a.

Method	Principal
Culture Dependent Genotyping Methods	
Random Amplified Polymorphic DNA - RAPD	Uses short arbitrary primers and low-stringency hybridization to randomly amplify DNA fragments which are separated to give a fingerprint pattern
Restriction Fragment Length Polymorphisms - RFLP	A profiling tool based on digestion of amplified ribosomal DNA using one or more restriction enzymes. Another name for this method is, ARDRA – Amplified Ribosomal DNA Restriction Analysis.
Culture Independent Molecular Methods	
Denaturing or Temporal Temperature Gradient Gel Electrophoresis - DGGE or TTGE	The separation of small PCR amplicons, distinguished by differences in their DNA sequences. Amplicons are separated from a low to high gradient, in the direction of the electrophoresis. DGGE uses a chemical gradient (urea or formamide). TGGE has a temperature gradient and a constant concentration of denaturants
Single Stranded Conformation Polymorphisms - SSCP	Allows separation of different DNA fragments of similar length on the basis of conformational differences in folded single stranded products and visualised on gels or as peaks using an automated sequencer
Real-Time PCR – qPCR	Uses a fluorescent probe to monitor amplification of the target DNA in real-time and enables quantification of a target species. Uses species-specific primers to target a gene/organism
Intergenic Transcribed Spacer Analysis – ITS	Analyses the bacterial ITS region located between the 16S and 23S ribosomal genes allowing differentiation between strains of the same species or closely related species.
Automated Ribosomal Intergenic Spacer Analysis - ARISA	A similar method to ITS but uses a fluorescent primer in the amplification of microbial ribosomal intergenic spacers. It generates peaks which correspond to discrete DNA fragments detected by a fluorescence detection system
Terminal Restriction Fragment Length Polymorphisms – T-RFLP	Based on the digestion of fluorescent, end-labelled, PCR products with restriction endonucleases after electrophoretic separation, the end-labelled terminal restriction fragments are compared with DNA size standards. Variation in the presence and location of the restriction sites result in different groups having different fragment lengths
Fluorescence In Situ Hybridization – FISH	Bacterial cells hybridize to a fluorescently labelled DNA probe and can be detected and counted by fluorescence microscopy techniques
Denaturing High Performance Liquid Chromatography – DHPLC	Separates PCR amplicons using an ion-pair revered-phase high performance liquid chromatography automated detection system.
Length Heterogeneity – PCR	Employs a Fluorescently labelled oligonucleotide as the forward primer, coupled with an unlabelled reverse pair to amplify hyper-variable regions. Labelled fragments are separated and detected by fluorescence with an automated sequencer.

Table 2: Comparison of studies employing both culture-dependent and culture-independent techniques for the analysis of microbial communities of raw milk and cheese.

Authors	Method	Culture Dependent Microorganisms	Culture Independent Microorganisms	Substrate
Randazzo et al., 2002	DGGE	*Lactococcus lactis* *Leuconostoc mesenteroides*[a] *Lactobacillus fermentum/ plantarum/ casei,* [a] *Pediococcus acidilactici*[a] *Enterococcus sulfurans/ faecalis* *Streptococcus thermophilus*[a] *Enterococcus hirae*[a]	*Lactococcus lactis* *Leuconostoc mesenteroides* *Lactobacillus fermentum/ plantarum/ casei/ delbrueckii* subsp*. bulgaricus* *Pediococcus acidilactici* *Enterococcus faecalis/ hirae/ sulfurans* *Streptococcus thermophilus/ bovis* *Macrococcus caseolyticus*	Cow's milk cheese
Cocolin et al., 2002	DGGE	*Candida catenulata/ pararugosa/ parapsilosis/ zeylanoides/ pseudointermedia/ rugosa* *Cryptococcus curvatus* *Kluyveromyces marxianus/ lactis* *Pichia guilliermondii* *Saccharomyces cerevisiae* *Trichosporon mucoides*	*Candida pseudorugosa/ kefyr/ pseudointermedia/ humulis/ rugosa* *Galactomyces spp.* *Kluyveromyces marxianus/ lactis* *Saccharomyces bayanus/ cerevisiae*	Cow's milk
Henri-Dubernet et al., 2004	TTGE	*Lactobacillus paracasei subsp. paracasei/ delbrueckii subsp. bulgaricus/ delbrueckii subsp. lactis/ casei subsp. casei/ acidophilus/ plantarum*[b]	*Lactobacillus paracasei subsp. paracasei/ plantarum*	Cow's milk cheese
Feurer et al., 2004	SSCP	*Arthrobacter arilaitensis* *Streptococcus thermophilus* *Lactococcus lactis subsp. lactis* *Brevibacterium linens* *Carnobacterium maltaromaticum* *Marine bacterium* *Corynebacterium casei* *Lactobacillus curvatus subsp. curvatus* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense* *Brachybacterium species*	*Arthrobacter arilaitensis* *Streptococcus thermophilus* *Lactococcus lactis subsp. lactis/ lactis* subsp*. cremoris* *Brevibacterium linens* *Carnobacterium maltaromaticum* Marine bacterium *Corynebacterium casei* *Lactobacillus curvatus subsp. curvatus, sakei* *Marinolactibacillus psychrotolerans* *Microbacterium gubbeenense* *Brachybacterium* species *Pseudoalteromonas* species Uncultured Flavobacterium	Cow's milk cheese
Callon et al., 2006	SSCP	*Kluyveromyces lactis/ marxianus* *Kluyveromyces* *Candida zeylanoides/ parapsilosis/ silvae/ intermedia/ tropicalis/ rugosa* *Debaryomyces hansenii* *Saccharomyces cerevisiae/ unisporus* *Pichia guilliermondii*	*Kluyveromyces lactis/ marxianus* *Kluyveromyces* *Candida zeylanoides/ parapsilosis/ silvae/ intermedia* *Debaryomyces hansenii* *Saccharomyces cerevisiae/ unisporus*	Cow's milk cheese

Table 2 continued:

Authors	Method	Culture Dependent Microorganisms	Culture Independent Microorganisms	Substrate
		Brachybacterium species *Enterococcus faecalis* [c] *Bacillus pumilus* *Staphylococcus pasteuri/ haemolyticus* *Lactobacillus casei* *Streptococcus dysgalactiae/ thermophilus* [c] *Lactococcus lactis* [c] *Lactococcus garvieae* *Microbacterium oxydans/ lacticum/ laevaniformans* *Sphingomonas* species *Chryseobacterium* species [c] *Flavobacterium* species [c] *Luteibactor rhizovicinus* [c] *Psychrobacter* species *Moraxella osloensis* *Stenotrophomonas maltophilia* [c] *Brevibacterium linens* [c] *Kocuria rhizophila/ carniphila* [c] *Arthrobacter arilaitensis* [c] *Corynebacterium flavescens* [c] *Staphylococcus fleurettii/ saprophyticus/ vitulinus/ epidermidis/ equorum/ pasteuri* [c] *Aerococcus viridans* [c] *Marinilactibacillus psychrotolerans* [c] *Brevundimonas nasdae* *Enterobacter agglomerans* [c] *Klebsiella oxytoca/ terrigena/ trevisani* [c] *Luteibactor rhizovicinus* [c] *Psychrobacter faecalis* *Enterobacter aerogenes* *Citrobacter freundii* *Moraxella osloensis* [c] *Streptococcus parauberis*	*Kocuria rhizophila* *Corynebacterium* species *Microbacterium foliorum* *Dietzia maris* *Dietzia* sp. *Nocardioides dubius* *Corynebacterium confusum* *Arthrobacter psychrolactophilus* *Leucobacter komagatae* *Corynebacterium xerosis* *Clostridium glycolicum/ lituseburense* *Enterococcus faecalis* *Staphylococcus warneri/ equorum* *Turicibacter sanguinis* *Jeotgalicoccus psychrophilus* *Lactobacillus casei/ kefiranofaciens* *Streptococcus dysgalactiae/ thermophilus* *Facklamia tabacinasalis* *Lactococcus lactis* *Mesorhizobium amorphae* *Bradyrhizobium japonicum* *Enterobacter agglomerans* *Ralstonia pickettii* *Alcaligenes* sp. *Acinetobacter lwoffii* *Chryseobacterium* sp. *Sphingobacterium* sp.	
Callon et al., 2007	SSCP	*Staphylococcus epidermidis/ simulans/ caprae/ equorum* *Kocuria rhizophila/ Kristinae carniphila* *Bacillus thuringiensis-cereus* *Micrococcus* species *Brevibacterium stationis* *Microbacterium oxydans* *Exiguobacterium* *Corynebacterium variable* *Brachybacterium paraconglomeratum* *Arthrobacter sp.* *Salinicoccus sp.* *Jeogalicoccus psychrophiles* *Micrococcus caseolyticus* *Ornithinicoccus sp.* *Dietza maris* *Rothia sp.* *Clostridium* *Enterococcus faecalis*	*Staphylococcus epidermidis/ caprae/ simulans/ equorum/* *Kocuria rhizophila/ Kristinae carniphila* *Bacillus thuringiensis-cereus* *Micrococcus* species *Brevibacterium stationis* *Microbacterium oxydans* *Exiguobacterium* *Corynebacterium variable* *Brevibacterium stationis* *Brachybacterium paraconglomeratum* *Arthrobacter* species *Salinicoccus* species *Jeogalicoccus psychrophiles* *Micrococcus caseolyticus* *Ornithinicoccus* species *Dietza maris* *Rothia* species *Enterococcus faecalis/ saccharominimus*	Goat's milk

Table 2 continued:

Authors	Method	Culture Dependent Microorganisms	Culture Independent Microorganisms	Substrate
		Lactobacillus casei *Leuconostoc mesenteroides* *Streptococcus mitis* *Enterococcus saccharominimus* *Pantoea agglomerans* *Pseudomonas putida/ aeruginosa/ fulgida* *Acinetobacter baumanii* *Citrobacter freundii* *Stenotrophomonas maltophilia* *Chryseobacterium indologenes* *Delftia acidovorans* *Enterobacter* species/*absuria* *Hahella chejuensis* *Klebsiella milletis-oxytoca* *Pseudomonas* *Candida* *Cryptococcus* *Debaryomyces* *Kluyveromyces* *Rhodotorula* *Trichosporon*	*Lactobacillus casei* *Leuconostoc mesenteroides* *Streptococcus mitis* *Pantoea agglomerans* *Pseudomonas putida/ aeruginosa* *Acinetobacter baumanii* *Citrobacter freundii* *Stenotrophomonas maltophilia* *Chryseobacterium indologenes* *Delftia acidovorans* *Enterobacter sp./ absuria* *Hahella chejuinsis* *Klebsiella milletis-oxytoca* *Candida* *Cryptococcus* *Debaryomyces* *Kluyveromyces* *Rhodotorula* *Trichosporon*	
Van Hoorde et al., 2008	DGGE	*Lactococcus lactis subsp. lactis*[a] *Lactobacillus paracasei/ plantarum/ brevis/curvatus* [a] *Pediococcus pentosaceus*[a] *Lactobacillus rhamnosus/ perolens* *Streptococcus salivarius* *Weissella paramesenteroides*	*Lactococcus lactis subsp. lactis* *Lactobacillus paracasei/ plantarum/ brevis/ curvatus/ rhamnosus/ parabuchneri/ gallinarum* *Pediococcus pentosaceus* *Enterococcus faecalis*	Cow's milk cheese
Nikolic et al., 2008	DGGE	*Lactobacillus paracasei* subsp. *paracasei* *Lactococcus lactis* subsp. *lactis* *Enterococcus faecalis*	*Lactobacillus paracasei* subsp. *paracasei* *Lactococcus lactis* subsp. *lactis* *Enterococcus faecalis* *Leuconostoc mesenteroides*	Goat's milk cheese
Gala et al., 2008	DGGE	*Lactobacillus casei/ buchneri*[a] *Lactobacillus paracasei* subsp. *paracasei/ tolerans/ rhamnosus* *Pediococcus acidilactici*[a]	*Lactobacillus casei/ delbrueckii* subsp*. lactis/ parabuchneri/ fermentum/ rhamnosus*	Cow's milk cheese
Dolci et al., 2008	DGGE	*Lactococcus lactis* subsp. *lactis/* subsp. *cremoris/* *Lactococcus lactis* *Lactobacillus plantarum/ paracasei/ casei/ coryneformis* subsp. *torquens/ delbrueckii* subsp. *lactis* *Enterococcus faecium* *Enterococcus faecalis*	*Lactococcus lactis* subsp. *lactis/* subsp. *cremoris* *Lactococcus lactis* *Lactobacillus plantarum/ kefiranofaciens* *Macrococcus caseolyticus* *Streptococcus agalactiae*	Cow's milk cheese

Table 2 continued:

Authors	Method	Culture Dependent Microorganisms	Culture Independent Microorganisms	Substrate
Aponte et al., 2008	DGGE	*Lactococcus lactis*[a] *Streptococcus thermophilus*[a] *Enterococcus faecalis/ durans/ faecium*[a] *Streptococcus parauberis* *Streptococcus haemolytics/croceolyticus/ warneri/pasteuri*[a] *Shigella boydii*[a] *Staphylococcus aureus*[a] *Macrococcus caseolyticus*[a] *Lactobacillus helveticus/ delbrueckii subsp. lactis/ delbrueckii subsp. bulgaricus/ delbrueckii subsp. delbrueckii/ delbrueckii subsp. indicus/ fermentum*[a] *Lactobacillus paracasei/ rhamnosus* *Lactococcus garvieae* *Leuconostoc mesenteroides subsp. lactis*[a] *Pediococcus acidilactici*[a]	*Lactococcus lactis* *Streptococcus thermophilus* *Macrococcus caseolyticus* *Morexella osloensis* *Weisella species* *Lactobacillus helveticus/ delbrueckii subsp. lactis* *Rahnella species* *Aeromonas simiae* *Staphylococcus aureus/ haemolyticus*	Cow's milk cheese
Abriouel et al., 2008	TTGE	*Lactobacillus paracasei/ plantarum/ brevis* *Lactococcus lactis* *Leuconostoc mesenteroides/ pseudomesenteroides* *Enterococcus devriesei/ faecium* *Enterococcus* *Pediococcus urinaequi* *Hafnia alvei* *Escherichia coli* *Obesumbacterium proteus* *Shigella flexneri*	*Lactobacillus plantarum/ brevis/ acidophilus/ paracasei* *Enterococcus species* *Lactococcus lactis* *Escherichia coli* *Nitrogen-fixing bacterium*	Goat's and sheep's milk cheese
Rantsiou et al., 2008	DGGE	*Lactobacillus planatrum/ brevis/ coryniformis/ paraplantarum* *Kluyveromyces lactis* *Pichia membranifaciens/ fermentans* *Candida krisii/ zeylanoides*	*Streptococcus thermophilus* *Lactobacillus delbrueckii* subsp. *bulgaricus /plantarum/ helveticus/suntoryeus/gallinarium* *Lactococcus lactis* *Kluyveromyces lactis* *Pichia membranifaciens/ fermentans* *Candida zeylanoides*	
Serhan et al., 2009	DGGE	*Lactobacillus curvatus/ plantarum* *Lactobacillus* *Enterococcus faecium/ durans/ faecalis/ malodoratus* *Lactococcus lactis* subsp. *lactis* *Streptococcus thermophilus*	*Lactobacillus plantarum* *Enterococcus faecium/ durans/ faecalis/ malodoratus* *Lactococcus lactis* subsp. *lactis* *Staphylococcus haemolyticus* *Streptococcus species* *Escherichia coli* *Clostridium bifermetnus/ Eubacterium tenue* Unidentified bands	Goat's milk cheese

Table 2 continued:

Authors	Method	Culture Dependent Microorganisms	Culture Independent Microorganisms	Substrate
Randazzo et al., 2009	DGGE	*Lactobacillus rhamnosus/ brevis* *Leuconostoc mesenteroides* *Lactococcus lactis* *Streptococcus thermophilus* *Enterococcus faecalis*	*Lactobacillus rhamnosus/ brevis/ plantarum/ pentosus/ fermentum/ buchneri/ delbrueckii* *Leuconostoc mesenteroides* *Lactococcus lactis* *Streptococcus thermophilus* *Enterococcus faecalis*	Sheep's milk cheese
Alegria et al., 2009	DGGE	*Lactococcus lactis/ garvieae* *Staphylococcus saprophyticus/ pasteuri* *Klebsiella species* *Lactobacillus plantarum* *Escherichia coli* *Micrococcus luteus* *Corynebacterium variable* *Flavobacterium species* *Leuconostoc mesenteroides* *Microbacterium oxydans* *Musa acuminata*	*Streptococcus thermophilus* *Lactococcus lactis/garvieae* *Streptococcus parauberis/ uberis/iniae* *Lactobacillus plantarum/ casei/paracasei* *Enterococcus faecium* *Corynebacterium variable* *Macrococcus caseolyticus* *Geotrichum candidum* *Kluyveromyces sp.* *Saccharomyces sp.* *Trichosporon gracile*	Cow's milk cheese
Alessandria et al., 2010	DGGE	*Lactococcus* subsp. *lactis/ garvieae* *Enterococcus faecium/ casseliflavus/ faecalis/ italicus/ durans* *Lactobacillus pentosus/ brevis/ plantarum* *Staphylococcus capitis/ parauberis/ epidermis* *Macrococcus caseolyticus* *Leuoconostoc citreum/ mesenteroides* *Pediococcus pentosaceus* *Weissella paramesenteroides* *Candida paraugosa/ zeylanoides/ parapsilosis* *Aerobasidium pullulans* *Cryptococcus sp.* *Discophareina fagi* *Rhodotorula glutinis* *Debaromyces hansenii* *Trichosporon coremiiforme*	*Lactococcus lactis* subsp. *lactis* *Lactobacillus helveticus* *Leuconostoc pseudomesenteroides* *Delphinelaa strobiligena* *Escherichia coli* *Propionibacterium acnes* *Methylobacterium sp.* *Gluconobacter thailandicus* *Moraxella oslonesis* *Kocuria rhizophila* *Klebsiella sp.* *Aureobasidium pullulans* *Phoma herbarum* *Seyrigia humbertii* *Saccharomyces cerevisiae* *Filobasidium/Crytococcus sp.* *Candida pararugosa* *Rhizomucor miehei* *Alternaria alternata*	Goat's milk cheese
Dolci et al., 2010	DGGE	*Lactococcus lactis* subsp. *lactis/* subsp. *cremoris*[a] *Lactococcus lactis* *Lactobacillus casei/ plantarum/ coryneformis subsp. torquens/ acidipiscis*[a] *Streptococcus agalactiae*[a] *Streptococcus thermophilus*[a]	*Lactococcus lactis* subsp. *lactis/* subsp. *cremoris* *Lactobacillus casei/ helveticus* *Streptococcus agalactiae* *Spingomonas species*	Raw cow's milk cheese

Subscript a, b, c indicates that DGGE, TTGE or SSCP, respectively, were also used in identifying these isolates.

Table 3: DGGE profile results of bacterial and yeast microflora of artisanal cheeses compared to industrial cheeses (Bonetta et al., 2008).

Microorganisms	Artisanal Samples	Industrial Samples
Bacteria	*Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* *Streptococcus* species *Lactococcus garvieae* *Streptococcus parauberis* *Streptococcus macedonicus*	*Lactococcus lactis* subsp. *lactis* *Lactococcus lactis* subsp. *cremoris* *Streptococcus* species
Yeasts	*Geotrichum* species *Kluyveromyces lactis* *Candida sake* *Saccharomyces exigus* *Saccharomyces silvae* *Yarrowia lipolytica* *Candida catenulate*	*Geotrichum* species *Kluyveromyces lactis* *Candida sake* *Penicillium* species

Table 4: Changes observed in milk microbiota during refrigeration at 4°C for 24 hours, monitored by band intensity from DGGE/TTGE profile of one milk sample (Lafarge et al., 2004).

Bacteria Detected	Milk + 24 hr refrigeration
Lactococcus lactis	-
Lactobacillus species	D
Listeria species	+
Pseudomonas species	+
Streptococcus uberis	+
Klebsiella pneumoniae	-
Escherichia coli	D
Enterobacter species	-
Serratia marcescens	=
Brevibacterium linens	D
Propionibacterium acidipropionici	-
Staphylococcus species	+
Kocuria species	-
Propionibacterium jensenii/thoenii	A

A, appearance; D, disappearance; + increased intensity; -, decreased intensity; =, same intensity.

Literature Review II

The complex microbiota of raw milk

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Abstract

Here we review what is known about the microorganisms present in raw milk, including milk from cows, sheep, goats and humans. Milk, due to its high nutritional content, can support a rich microbiota. These microbes enter milk from a variety of sources and, once in milk, can play a number of roles, such as facilitating dairy fermentations (e.g. *Lactococcus*, *Lactobacillus*, *Streptococcus*, *Propionibacterium* and fungal populations), causing spoilage (e.g. *Pseudomonas*, *Clostridium*, *Bacillus* and other spore-forming or thermoduric microorganisms), promoting health (e.g. lactobacilli and bifidobacteria) or causing disease (e.g. *Listeria*, *Salmonella*, *Escherichia coli*, *Campylobacter* and mycotoxin producing fungi). There is also concern that the presence of antibiotic residues in milk leads to the development of resistance, particularly among pathogenic bacteria. Here we comprehensively review these topics, while comparing the approaches, both culture-dependent and -independent, that can be taken to investigate the microbial composition of milk.

1. Introduction

Milk is a highly nutritious food that can be obtained from a variety of animal sources such as cows, goats, sheep and buffalo, as well as humans, for human consumption. However, the high nutrient content of these milks, which includes proteins, fats, carbohydrates, vitamins, minerals and essential amino acids (see Supplementary Table S1), all at a near neutral pH and at a high water activity, provides an ideal environment for the growth of many microorganisms. Some of these nutrients are directly available to all microorganisms, while others are provided following the metabolism of major components by specific populations to release components and metabolites that are used by others (Frank, 1997). It is generally accepted that the lactic acid bacteria (LAB), a group of bacteria which ferment lactose to lactate, are a dominant population in bovine, goat, sheep and buffalo milk, prior to pasteurisation. The most common LAB genera in milk include *Lactococcus, Lactobacillus, Leuconostoc*, *Streptococcus* and *Enterococcus*. Psychrotrophic populations, which particularly establish themselves during cold storage, are also a major component and frequently include *Pseudomonas* and *Acinetobacter* spp. Other strains of non-LAB genera are also encountered in milk, as well as various yeasts and moulds (Quigley et al., 2011). Human milk on the other hand is typically dominated by *Streptococcus, Staphylococcus, Lactobacillus* and *Bifidobacterium* spp. (Martín et al., 2007).

The specific composition of the milk microbiota directly impacts on the subsequent development of dairy products (Figure 1). Microorganisms can bring about the fermentation of milk through the production of lactate and have a variety of different impacts on the sensory, texture, flavour and organoleptic properties of resultant products (Wouters et al., 2002). Microorganisms can also negatively impact on milk quality and shelf life; for example, psychrotolerant bacteria can proliferate during refrigeration and, through the production of extracellular lipases and proteases, result in spoilage (Desmasures and Gueguen, 1997, Hantsis-Zacharov and Halpern, 2007). The microbial composition of milk can also have health- related implications in that the consumption of raw milk contaminated with pathogens can lead to, in some cases severe, illness (Oliver et al., 2009). In contrast, it is

claimed that other raw milk microbes can contribute to health by aiding digestion or reducing the frequency of allergies, including asthma and atopic diseases, in individuals who consume raw milk during the early years of life (Debarry et al., 2007, Braun-Fahrländer and Von Mutius, 2011). This review will highlight the various microbial populations found in raw milk and the methods employed for their detection. It also addresses their sources, their subsequent significance with respect to industrial applications and the contribution of specific populations to food quality and health.

2. Methods employed to determine the microbial composition of milk

Many microbial communities are complex, *i.e.* they are comprised of many different taxonomical groups of microbes. Raw milk is an example of an environment that contains a diverse and complex microbial population (Quigley et al., 2011, Vacheyrou et al., 2011). Most of our knowledge with respect to the identity of the microbes that are present in raw milk, and resultant dairy products, has been gained through the growth or "culturing", and subsequent analysis, of these microorganisms. The ultimate identification of these cultured microorganisms involves phenotypic and/or genotypic methods. Phenotypic methods are those which have been traditionally employed and involve the growth of microorganisms in microbiological media (either general or selective) supplemented with morphological, biochemical or physiological characterisation (Quigley et al., 2011). These testing methods are still the standard in industrial settings and typically involve tests to determine total bacteria counts, reflecting general milk quality, or detect specific pathogens or other microorganisms which indicate if contamination has occurred. Populations frequently tested for include thermoduric populations (resisting pasteurization), sulfate reducing clostridia, *Listeria monocytogenes, Salmonella,* coagulase-positive staphylococci*, Escherichia coli, Enterobacteriaceae*, coliforms and *Bacillus cereus* among others. These standard methods are legislated and accredited by National and International Accreditation Boards (e.g. http://eur-lex.europa.eu; http://www.inab.ie/). These tests generally rely heavily on the use of microbiological broths or agars that

selectively support the growth of the target microbial population and often include further confirmatory biochemical analysis. These approaches are usually low tech and inexpensive but are relatively labour intensive and time consuming and, in some cases, insufficient discriminatory power can be a problem. More recently, considerable efforts have been made to develop more rapid, high-throughput tests that rely on DNA-based, genotypic analysis. Such technologies, which usually rely, at least to some extent, on the application of Polymerase Chain Reaction (PCR) technology, can be used to confirm the results generated through traditional tests, but their ability to serve as an alternative to culture-based analysis is increasingly being appreciated. One of the key benefits of replacing the culturing step relates to the fact that many microorganisms are averse to isolation using common culturing methods, thus potentially leading to a significant underestimation of the microbial communities. A number of factors must be considered when applying these culture-independent methods. The selection of a protocol that efficiently extracts nucleic acids from as many of the microorganisms present as possible is critical. One must also consider the use of strategies, such as the use of DNA-binding agents or an alternative focus on RNA, to limit the risk of false positives resulting from the amplification of DNA from dead cells (Quigley et al., 2011). Finally, a decision has to be made regarding the genes or genes to be targeted. The oligonucleotides or probes can be selected to detect target-specific genes or to provide an overview of the microbiota within a particular niche through the non-target specific amplification of highly conserved genes, such as the 16S or 23S rRNA genes. In the latter case, amplified products can then be analysed by techniques such as denaturing gradient gel electrophoresis (DGGE), temporal temperature gradient gel electrophoresis (TTGE) or single-stranded conformation polymorphism (SSCP) (Quigley et al., 2011) to highlight similarities or differences in the populations. These approaches may be used in conjunction with Sanger (first generation) DNA sequencing, to help specifically identify the populations present. More recently, there has been a rapid evolution in next-generation DNA sequencing technologies that produce millions of sequence reads in a single run, thus allowing a much more in-depth and accurate estimation of microbial diversity. The ever increasing number and length of the sequence

reads provided by these technologies, coupled with the availability of databases and bioinformatic tools has been hugely beneficial with respect to the taxonomic assignment of the microbes present (Loman et al., 2012). Although, to date, high-throughput sequencing approaches have not been extensively applied to assess the microbiota of dairy-based environments, there have been a number of recent publications which suggest that this situation will change dramatically in the coming years (Alegria et al., 2012, Masoud et al., 2011, Masoud et al., 2012, Quigley et al., 2012).

3. Sources of milk microorganisms

Milk in healthy udder cells is thought to be sterile (Tolle, 1980) but thereafter becomes colonised by microorganisms from a variety of sources, including the teat apex, milking equipment, air, water, feed, grass, soil and other environments (Figure 1) (Coorevits et al., 2008, Lejeune and Rajala-Schultz, 2009, Vacheyrou et al., 2011).

The bovine teat surface can contain a high diversity of bacteria (Braem et al., 2012, Monsallier et al., 2012, Verdier-Metz et al., 2012). In one particularly detailed study, culture-dependent methods revealed that the bacteria present could be classified at the phylum level as Firmicutes (76%) Actinobacteria (4.9%), Proteobacteria (17.8%) and Bacteroides (1.3%). When this approach was supplemented by a clone-library sequencing-based approach, some additional phyla, *i.e.* Planctomycetes, Verrucomicrobia, Cyanobacteria, Chloroflexia, and unclassified Bacteria, were detected at low levels (Verdier-Metz et al., 2012). Notably, a large percentage of the reads from this and other studies (Fricker et al., 2011) corresponded to as yet unidentified bacteria. Of those that could be identified, many corresponded to technologically important bacteria such as *Lactobacillus, Leuconostoc* and *Enterococcus* spp*.* Bacteria that can be involved in flavour, aroma and colour development in cheese such as coagulase-negative staphylococci, as well as *Arthrobacter, Brevibacterium* and *Corynebacterium* spp. were also detected. However, some of the microorganisms detected on the teat surface, *e.g. Solobacterium, Clavibacter* and *Arcanobacterium* spp., have not been identified in milk (Verdier-Metz et al., 2012), presumably reflecting a lack of

competitiveness in milk environments should transfer occur. It was also noted that the composition of the microbial community on the teat surface varied qualitatively and quantitatively from one farm to another (Verdier-Metz et al., 2012). This can be attributed to many different factors, for example microorganisms associated with bedding material can contaminate the surface of teat and thus potentially enter milk (Vacheyrou et al., 2011). Similarly, milking machines can contain a reservoir of microorganisms and thus, unsurprisingly, differences between machines and related practices can influence the microbial population of the milk collected (Michel et al., 2006). With respect to more general environmental factors, it has been observed that the microorganisms present in cow's milk depend on whether animals are fed indoors or outdoors, with an increase of *Staphylococcus* spp. during outdoor feeding (Hagi et al., 2010), on the location of the animals (Bonizzi et al., 2009) and on the lactation stage (Callon et al., 2007). An intense study was carried out to relate the microorganisms detected in milk with where they can be found on the farm (Vacheyrou et al., 2011). These results highlighted 141 bacterial species, representing 54 genera, from throughout the farm. There were 25 genera detected in these milk samples and many of these, including *Aerococcus, Streptococcus, Propionibacterium, Acinetobacter, Bacillus, Ochrobactrum, Pseudomonas, Psychrobacter, Staphylococcus, Sphingomonas, Enterobacter, Pantoea, Brachybacterium, Corynebacterium, Kocuria, Microbacterium* and *Pseudoclavibacter*, were also detected in different areas throughout the farm including teat surfaces, milking parlours, hay, air and dust. Also present in milk, but not detected in the farm environment, were technologically relevant bacteria such as *Lactococcus, Lactobacillus,* and *Enterococcus* as well as *Leucobacter, Deinococcus* and *Paracoccus*. Similarly, a large number of other taxa were detected in the farm environment but not in milk (Vacheyrou et al., 2011). Finally, it is notable that the implementation of strict hygiene standards brings about a reduction in microbial load of milk, including a reduction in populations of technological importance which can, in turn, impact negatively on cheese manufactured using traditional or artisanal approaches (Monsallier et al., 2012). Indeed, Mallet et al. (2012) recently reported a one-magnitude reduction in the levels of technologically relevant lactococci present in raw milk relative to what had

been detected 15 years before in raw milk collected from the same area (Desmasures and Gueguen, 1997). These populations seem to be particularly sensitive to the evolution of farm practices, as other populations, such as *Pseudomonas*, *Lactobacillus* and yeast populations, did not differ across the two studies. While it is important to ensure that the quality of milk is maintained at high levels, producers of traditionally manufactured raw milk cheese should be aware that certain farming practices may negatively impact on distinctive flavours and aromas as a consequence of limiting the numbers of specific microorganisms and may need to compensate through the introduction of starters and adjunct strains.

4. The microbial composition of different milk types

Although the largest proportion of commercially produced milk worldwide comes from cows, there are a number of other animal sources of milk that is used for human consumption. These include quite common sources such as goat, sheep, buffalo and others utilised in more specific regions such as camel milk in African and Arab countries and yak milk in Asian countries. This section will review recent findings on the microbial content of these various milks. We will also discuss an issue that has been receiving ever more attention in recent years, *i.e.* the microbial composition of the human milk that is consumed by infants only.

4.1 Cow's Milk

Cow's milk is produced on a massive scale. In 2012 the EU produced ~139 million tonnes of cow's milk followed by the US with 90 million tonnes (http://www.dairyco.org.uk/market-information/supply-production/milk-production/world-milk-production/). This milk is employed in many ways, including direct consumption and the manufacture of dairy products and milk powders. Raw cow's milk has the potential to contain a diverse bacterial population as highlighted previously (Quigley et al., 2011). Typically cow's milk contains a significant LAB population that includes *Lactococcus* ($8.2x10^1$ – $1.4x10^4$ cfu ml^{-1}), *Streptococcus* ($1.41x10^1$ – $1.5x10^4$ cfu ml^{-1}), *Lactobacillus* ($1.0x10^2$ – $3.2x10^4$ cfu ml^{-1}), *Leuconostoc* ($9.8x10^1$ - $2.5x10^3$ cfu ml^{-1}) and

Enterococcus spp. ($2.57x10^1$ – $1.58x10^3$ cfu ml^{-1}) (Figure 2). A number of other microbes can be present in significant proportions. These include psychrotrophs, such as *Pseudomonas, Acinetobacter* and *Aeromonas* spp., which flourish during cold storage (Raats et al., 2011). However, while the bacterial composition of cow's milk has been extensively studied for quite some time, new developments with respect to DNA sequencing technologies have highlighted that the diversity of these bacteria is greater than originally appreciated (Table 1). Indeed, a recent study applied high-throughput DNA sequencing to examine the bacterial population of raw cow's milk that was to be used for cheese production (Masoud et al., 2012); 256 bacterial species were detected, of which *Streptococcus thermophilus* and *Lactococcus lactis* dominated in the milk, representing 43.7% and 19% of reads, respectively. A number of other microbes that had previously been associated with raw milk, including *Acinetobacter, Aeromonas, Brevibacterium, Corynebacterium, Lactobacillus, Pseudoalteromonas, Pseudomonas* and *Staphylococcus*, which represented between 1.3 - 3.7% of the total reads, were also detected. A large sub-population of taxa, which each corresponded to <1% of the total reads, was also highlighted (Masoud et al., 2012). We have also recently compared the bacterial population present in cow's milk pre- and post-pasteurisation using high-throughput sequencing to reveal the presence of a previously unrecognised and diverse bacterial population in unpasteurised cow's milk. While the milk, sourced from a variety of commercial producers throughout Ireland, was dominated by *Lactococcus*, *Pseudomonas* and *Leuconostoc*, we also detected a number of anaerobic taxa, including *Bacteroides, Faecalibacterium, Prevotella* and *Catenibacterium*, which are more typically associated with the gut microbiota and may be entering the milk through faecal contamination. Our analyses indicate that the bacterial population of pasteurised milk is more diverse than previously appreciated but that the non-thermoduric bacteria that are present within these populations are likely to be in a damaged, non-culturable form (Quigley et al., 2013). Thus, high-throughput sequencing approaches can provide a detailed insight into the bacterial composition of milk and it is likely that these technologies will be used increasingly in the future to investigate the factors that influence the composition of cow's milk.

4.2 Goat's milk

Goat's milk production represents about 2.1% of global milk production (Tsakalidou and Odos, 2012). It is an important commodity that has gained increased interest as an alternative to cow's milk, due to evidence that it is less likely to induce allergies (Park, 1994). Goat's milk also differs from cows and sheep milk by virtue of having greater levels of iron bioavailability (Boyazoglu and Morand-Fehr, 2001) as well as containing smaller fat globules, having a higher content of fatty acids and forming a softer curd during subsequent fermentations, in turn leading to greater digestibility (Klinger and Rosenthal, 1997). Goat's milk is most frequently used for cheese making, usually at farm level or in small dairies. Goat's milk cheeses are particularly common in Mediterranean countries and South-East Europe (Pirisi et al., 2007). Goat's milk is also typically dominated by LAB, including species of *Lactococcus* ($3.7x10^6$ cfu ml^{-1}), *Lactobacillus* ($1.34x10^5$ cfu ml^{-1}), *Leuconostoc* ($3.27x10^3$ cfu ml^{-1}) and *Enterococcus* ($2.95x10^2$ cfu ml^{-1}), as well as *Enterobacteriaceae, Micrococcaceae,* moulds (filamentous fungi) and yeasts (Alonso-Calleja et al., 2002, Nikolic et al., 2008, Tamagnini et al., 2006). Callon *et al.* (2007) relied on the use of selective microbiological media, SSCP analysis as well as Restriction Fragment Length Polymorphism (RFLP) typing of isolates to examine the microbial diversity of 118 goat's milk samples taken from one herd throughout one lactation year to reveal the presence of a diverse bacterial population in the milk (Table 2). In addition to microorganisms commonly encountered in milk, such as those listed above, some species were identified that are not typically associated with goat's milk or that had previously only been associated with cheeses, including a number of corynebacteria and brachybacteria. Another unexpected finding was the presence of several halophilic species not previously associated with milk, including *Jeotgalicoccus psychrophilus*, *Salinicoccus* sp., *Dietzia maris*, *Exiguobacterium*, *Ornithinicoccus* sp. and *Hahella chejuensis*. The significance of the presence of these microorganisms with respect to health, safety or product development is not known. Through this approach it was also revealed that milks collected during winter were dominated by the presence of *Lactococcus* and *Pseudomonas*, those from summer by *Pantoea agglomerans* and *Klebsiella* and those from autumn by *Chryseobacterium*

indologenes, *Acinetobacter baumannii*, *Staphylococcus*, *Corynebacteria* and yeasts. While these variations can be attributed to differences in feed, the authors suggested that other factors, such as weather conditions and the health of the animal, were also important (Callon et al., 2007). There has not been an in-depth assessment of the microbiology of goat's milk since this study and next-generation sequencing technologies would have the potential to be very revealing.

4.3 Sheep's milk

Sheep's milk is rarely consumed but still constitutes ~1.3% of global milk production as it is often employed throughout Europe in the development of cheese (Tsakalidou and Odos, 2012). Sheep's milk is dominated by LAB, with mesophilic bacteria representing 10^2 to 10^6 cfu ml^{-1}, while psychrotrophic populations correspond to 10^2 to 10^4 cfu ml^{-1} (Fotou et al., 2011). Studies assessing the impact of storing sheep's milk at refrigeration temperature highlighted increases in psychrophiles but also in mesophiles. Unsurprisingly, the thermoduric population did not increase. These general trends are also affected by temperature and the length of storage (de Garnica et al., 2011). Other bacteria that have been detected on occasion can include microbes of concern from a milk safety perspective including *E. coli, Salmonella, Staphylococcus aureus, Bacillus* and *Clostridium perfringens* (Fotou et al., 2011). The location can affect both the nutritional composition and microbial composition of sheep's milk. A correlation has been noted between milks with a higher fat content and greater counts of LAB, coliforms and moulds. In populations of streptococci and *S. aureus*, there was a increase and decrease of counts, respectively, in regions where the milk was more acidic and nutrient levels were lower (Yabrir et al., 2013). Some insight into the microbiology of sheep's milk was also provided by a recent study of the raw sheep's milk cheese, Oscypek, which is manufactured without a starter culture (Alegria et al., 2012) (Table 3). As this is a naturally fermented raw milk cheese, it is likely that these cheese-associated bacteria were also present in the corresponding raw milk. A culture-based approach established that lactococci (*L. lactis* ssp. *lactis* and ssp. *cremoris*) dominated (~10^9 cfu g^{-1}), with lactobacilli (*L. casei, L. plantarum, L. parabuchneri* and *L. brevis*) also being

common (10^7 - 10^8 cfu g^{-1}). *Leuconostoc* (*Leuc. citreum, Leuc. lactis* and *Leuc. mesenteroides*) were detected at levels of 10^5 - 10^8 cfu g^{-1}, fungal populations were present between 10^5 - 10^6 cfu g^{-1} and Enterobacteriaceae, including *Enterobacter kobei*, were at 10^3 - 10^6 cfu g^{-1}, but were reduced during processing. A parallel DGGE investigation confirmed the dominance of *L. lactis* but also highlighted the presence of a significant population of *L. garvieae*, which had not been detected by culturing. This approach also revealed a number of minor populations including *Tetragenococcus halophilus, Streptococcus salivarius, S. thermophilus* and *S.tococcus vestibularis*. A high-throughput sequencing-based approach revealed the presence of 40 different genera in the cheese. This included 9 dominant genera, including 6 from the order Lactobacillales (which includes the lactococci, lactobacilli and related genera), which constituted 97% of assigned sequences. The other dominant genera were the *Bifidobacteriaceae*, *Enhydrobacter* and unclassified Bacilli (Figure 2). The benefits of employing this technology were again highlighted when previously overlooked populations of *Kocuria, Sanguibacter, Flavobacteria, Chryseobacterium, Exiguobacterium, Staphylococcus* and *Chromohalobacter* were detected. Notably, a considerable proportion, ~20%, of sequence reads could not be assigned and so the identity of these bacteria, and the importance of the other subpopulations, will require further attention (Alegria et al., 2012) (Table 3).

4.4 Buffalo milk

Buffalo milk is consumed in various countries around the world, with India and Pakistan being the highest consumers. It is not as common in Europe but it does have an important market in some Mediterranean countries where it is utilised in making traditional mozzarella cheese. The microbial content of raw buffalo milk has been assessed, through culturing, and found to contain a large population of LAB, including lactococci and lactobacilli, as well as coliforms, including *E. coli, S. aureus* and bacterial endospores, highlighting that while technologically relevant bacteria are present, microbes of concern with respect to quality and safety can also be found (Ercolini et al., 2004, Han et al., 2007). Culture-independent methods, i.e. DGGE, have revealed that raw buffalo milk contains a rich diversity of bacteria that changes during

subsequent fermentation to manufacture traditional mozzarella (Ercolini et al., 2001). More recently, high-throughput sequencing has been applied to identify the bacterial populations present in buffalo milk and throughout the manufacture of mozzarella cheese (Table 3) (Ercolini et al., 2012). The dominant microbes in the milk were *Lactococcus* spp. (30%), *Acinetobacter* spp. (21%), *Pseudomonas* spp. (20%), *S. macedonicus* (10%) and *L. lactis* (10%) (Figure 2). A number of other microbes were detected in low abundance including *Brochothrix, Carnobacterium, Chryseobacterium, Clostridium, Corynebacterium, Enterobacteriaceae, Gamma-proteobacteria* and *Haloanella.* There was also a large percentage of unassigned reads (~20%) corresponding to the raw milk. This percentage was much greater than that associated with the corresponding cheese (Ercolini et al., 2012).

4.5 Milk from other animal sources

Other milks which are consumed by humans around the world include those produced by camels, yaks, donkeys and, to a lesser extent and in only some countries, mares. Camel milk is commonly consumed in African and Arab countries where it is a valuable food resource for pastoral people. The microbial population of camel milk, like that of other milks, can play a role in subsequent fermentations, health promotion and milk spoilage. The milk is typically dominated by mesophiles, including lactobacilli (such as *L. helveticus*, *L. casei* ssp *casei* and *L. plantarum*, lactococci (such as *L. lactis* ssp. *lactis*), streptococci (such as *S. salivarius*) and leuconostoc (all at 10^2-10^7 cfu ml^{-1}) (Khedid et al., 2009). As other milks, camel milk can also contain human pathogens, including *Salmonella*, *Listeria* and *E. coli*, the prevalence of which can vary with location (Abeer et al., 2012). In naturally fermented camel milk, *Streptococcus infantarius* ssp. *infantarius* dominates, with significant populations of *L. lactis* ssp. *lactis*, *S. thermophilus* and *L. helveticus* also detected *(Jans et al., 2012)*. Another animal which is typically associated with difficult climates is the yak. ~92% of the world's yaks are located in China and yak milk can be an important commodity in some regions of China. Again the LAB dominate, with yeasts and coliforms also being common (Zhang et al., 2008a). Other studies have identified *L. casei, L. delbrueckii* ssp. *bulgaricus, L. fermentum, L. kefiranofaciens, L. plantarum* ssp. *plantarum, L.*

brevis, L. buchneri, Leuc. lactis, Leuc. mesenteroides, *L. lactis* ssp. *cremoris*, *S. thermophilus*, *Ent. faecalis, Ent. durans* and *Weisella cibaria* being among the most common species in yak milk (Watanabe et al., 2008, Yu et al., 2011). Donkey milk is less commonly consumed and, for the same reason, is less extensively studied. The microbial content of donkey milk has been found to be similar to that of other milks, consisting of LAB, coliforms and fungi, with mesophiles being detected at levels up to 10^4 cfu ml^{-1} and psychrotrophs at 10^2 cfu ml^{-1} (Sarno et al., 2012, Zhang et al., 2008b).

Box 1. Human milk

Human milk has the potential to protect a newborn against infectious disease through the provision of active components including immunoglobulins, immunocompetent cells, fatty acids, oligosaccharides and glycoproteins (Newburg, 2005, Saaverda, 2002). Furthermore, breast milk is also a source of microbes and of growth factors that contribute to their growth in the gut. Traditionally, it has been thought that these microorganisms are transferred from the mother's skin (West et al., 1979). However, recent studies have indicated that bacteria present in the maternal gut can reach the mammary gland by vertical transfer (Albesharat et al., 2011). This topic requires further investigation. Regardless of the origin of these microorganisms, human milk constitutes one of the primary sources of the bacteria that colonise the gut of breastfed infants. Indeed, an infant consuming approximately 800 ml day^{-1} of human milk would be predicted to ingest between 10^5 and 10^7 bacteria daily (Heikkila and Saris, 2003). This may explain why some studies have shown that the bacterial composition of the gut microbiota of breast-fed infants closely resembles that found in the breast milk of their mothers (Albesharat et al., 2011). As for other milks, a variety of different approaches have been taken to investigate the microbiology of human milk. Traditional culture-based methods have indicated that staphylococci, LAB and propionibacteria dominate in human milk with a significant *Bifidobacterium* population also present (Table 4) (Martin et al., 2009). Similarly, several studies have demonstrated the transfer of *Staphylococcus, Lactobacillus*, *Bifidobacterium* as well as *Enterococcus* spp. from mother to infant through breast-feeding

(Albesharat et al., 2011, Martin et al., 2009, Martin et al., 2004). The application of culture-independent molecular techniques, and particularly those based on analysis of 16S rRNA genes, has facilitated even more detailed analyses. These approaches have provided quite similar findings, *i.e.* a dominance of staphylococci and streptococci and the presence of LAB, propionibacteria and bifidobacteria. However, DNA from other bacterial groups, including *Weisella, Clostridium* and *Serratia*, was also detected (Table 4) (Martin et al., 2009, Martin et al., 2004). More recently, through the application of high-throughput sequencing, an even more diverse population has been uncovered (Table 4) (Cabrera-Rubio et al., 2012, Hunt et al., 2011). Some taxa that were consistently found across all samples, included species of *Streptococcus, Staphylococcus, Serratia, Pseudomonas, Corynebacterium, Ralstonia, Propionibacterium, Sphingomonas* and *Bradyhizobiaceae* (Figure 2) (Cabrera-Rubio et al., 2012, Hunt et al., 2011). The microbial composition of breast milk changed from pre-labour to post-birth. Where birth was by non-elective caesarean section the bacterial profile was more comparable with milk from mothers who had a natural labour, than that of milk from mothers who underwent elective caesarean section. These results suggest that the physiological changes in the mother during the labour process may influence the composition of the bacterial community. Finally, as a consequence of nursing periods, the bacterial population of breast milk eventually became dominated by microbes from the oral cavity and skin (Cabrera-Rubio et al., 2012). Following on from these recent investigations, it will be important to continue to unravel the roles of these individual microbial components with respect to mammary gland health, colonisation of the infant gut and, subsequently, maternal and infant health.

Importantly, it is already known that human milk bacteria can play important roles in the infant gut. They can contribute to a reduction of the incidence and severity of infections by different mechanisms through competitive exclusion, the production of antimicrobial compounds or improving intestinal barrier function (Olivares et al., 2006). Many human milk lactobacilli and bifidobacteria can contribute to infant digestion by aiding in the breakdown of complex foods such as proteins and sugars; some lactobacilli increase the production of functional metabolites such as butyrate, which is

utilised as an energy source and can improve intestinal function (Asakuma et al., 2011, Gil-Campos et al., 2012, Zivkovic et al., 2011), while various bifidobacteria have had positive effects on health, including prevention of infection by pathogenic bacteria (e.g. protection against diarrhea), immunostimulatory and anti-carcinogenic capabilities, lowering of serum cholesterol and alleviation of lactose maldigestion (Fernández et al., 2012).

While breast milk can introduce a number of potentially health-promoting bacteria, it may also contain pathogens (Jones, 2001). Mastitis can be a common disease, with incidence rates of up to 33% in lactating mothers (Foxman et al., 2002) (the issue of bovine mastitis is addressed later in the review). Mastitis results in the inflammation of the mammary lobules, usually due to the presence of staphylococci, streptococci or corynebacteria. Traditionally, *S. aureus* has been considered as the main causative agent; however, *S. epidermidis* is emerging as a leading cause of subacute and acute mastitis in both women and veterinary medicine (Delgado et al., 2009).

The possible presence of pathogenic or spoilage microorganisms in human breast milk also needs to be considered in the context that mothers may have to store milk for a variety of different reasons. Human milk may be stored at neonatal units and used as a life-saving therapy to high risk infants (Silvestre et al., 2006). The influence of refrigeration on the bacterial content of human milk, over a four-day period, has recently been assessed; refrigeration prevented the growth of total aerobic bacteria, LAB and *Enterobacteriaceae*, while reducing the levels of coagulase-positive staphylococci (Giribaldi et al., 2013).

5. Technologically relevant bacteria of raw milk

As described above, raw milk can contain a diverse bacterial population. Many such bacteria can contribute subsequently to natural fermentations. In some situations, specific strains have been so successful in this regard that they have been isolated from milk and consciously added as starters or adjuncts designed to confer desirable traits on fermented products. This can be particularly important in situations where regulations require the use of pasteurised milk and, thus, the re-introduction of dairy microorganisms can

compensate for the removal of commensal populations and the associated adverse affect on the flavour of resultant products. This section will review what is known about the most technologically important genera of raw milk bacteria.

5.1 *Lactococcus*

Lactococcus consists of seven species, two subspecies and one biovar (www.bacterio.cict.fr; as of November 2012). Of these, *Lactococcus lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* can dominate in raw milk, cheese and other (unheated) dairy products.

In dairy foods, *L. lactis*, and *L. lactis* ssp. *lactis* and *L. lactis* ssp. *cremoris* in particular, are primarily known for their role as starter cultures for the cheese industry. *L. lactis* ssp. *lactis* biovar *diacetylactis* is also recognised for its production of flavour developing compounds (Hugenholtz and Starrenburg, 1992). These microorganisms are distinguished from one another on the basis of arginine and citrate utilization, growth temperature and salt tolerance (Kahala et al., 2008). While these microorganisms are naturally present in raw milk and artisanally produced cheeses (Gaya et al., 2001), they are frequently added to pasteurised milk to facilitate the commercial manufacture of cheeses (Smit et al., 2005). Their primary role during cheese production is acidification through the production of L-lactate. However, they also contribute to proteolysis, the conversion of amino acids into flavour compounds (alcohols, ketones, aldehydes), citrate utilisation and/or fat metabolism (Smit et al., 2005). A comparison of 20 *L. lactis* strains, 10 of the ssp. *lactis* phenotype and 10 of the ssp. *cremoris* phenotype, confirmed two major subspecies lineages that were distinguished on the basis of the presence or absence of 4,571 gene orthologs (Bayjanov et al., 2009, Fernández et al., 2011). Thus, it is estimated that these phenotypically similar subspecies diverged approximately 17 million years ago (Bolotin et al., 2004). Sequencing of the genome of *L. lactis* ssp. *lactis* IL1403 revealed that all known genes required for energy metabolism were present, including a number of genes involved in fermentation as well as a novel gene, *poxL*, encoding pyruvate oxidase, which may play a role in switching between fermentation modes. 43 insertion elements were identified, the distribution of

which suggests that recent recombination between two closely related genomes may have occurred (Bolotin et al., 2001). Sequencing of *L. lactis* ssp. *cremoris* MG1363 revealed some similarities to strain IL1403, including proteolytic systems and genes associated with the utilisation of lactose. The absence of virulence genes from these genomes is consistent with their GRAS status (Wegmann et al., 2007). Genome sequencing of *L. lactis* ssp. *cremoris* strain A76 revealed that it has 99.2% identity to the industrially important strain *cremoris* SK11 and identified two contiguous regions associated with the ssp. *lactis* lineage. The first contains genes involved in cell wall biosynthesis. The second region corresponds to a prophage. The presence of these regions suggests that they were introduced through a recombination event and highlights the potential importance of such events among strains of industrial importance (Bolotin et al., 2012). The *L. lactis* ssp. *cremoris* SK11 strain contains a number of unique plasmids, pSK11A, pSK11B, pSK11L and pSK11P, which encode important traits related to dairy adaptation and utilisation, including lactose utilisation, a complex proteolytic system and an oligopeptide permease system (Siezen et al., 2005). *L. lactis* ssp. *lactis* biovar *diacetylactis* strain DPC 3901 contains four unique plasmids, pVF18, pVF21, pVF22 and pVF50. While sequence analysis of these plasmids has revealed that this bacterium most likely originated from a plant origin, there are some features which highlight its adaption to milk, e.g. plasmid pVF59, which contains genes of relevance for growth on milk. These include genes encoding a lactose phosphotransferase operon, a protein predicted to function in D-lactate utilisation, a system for uptake of oligopeptides generated from casein degradation as well as oligopeptidases, which allow this strain to utilise casein as a nitrogen source (Fallico et al., 2011).

A number of other species of *Lactococcus* are naturally present in raw milk. Although *L. raffinolactis* is typically not used by the dairy industry because of a lack of caseinolytic activity (Holler and Steele, 1995), a recent study observed synergism between *L. raffinolactis* and *L. lactis* strains, whereby *L. raffinolactis* improved acid production thanks to its ability to utilise metabolic products generated by *L. lactis* (Kimoto-Nira et al., 2012), presumably thanks to the presence of a complete set of genes for lactate

fermentation and genes responsible for an oligopeptide ABC transporter (Meslier et al., 2012a). Even though *L. garvieae* is a recognised fish pathogen (Vendrell et al., 2006), it has been detected in raw milk, some natural mixed starter cultures and artisanal cheeses (Fortina et al., 2007, Foschino et al., 2008). Genomic studies have shown that *L. garvieae* isolates from dairy and fish sources form two distinct clusters and that only the dairy isolates possess the ability to utilise lactose (Fortina et al., 2009, Fortina et al., 2007). It is hypothesized that this key phenotype has been gained by dairy isolates through lateral gene transfer (Ferrario et al., 2012). However, strains from both clusters lack proteolytic activity (Fortina et al., 2007). Finally, *L. piscium* has been detected in raw milk and raw milk cheese (Carraro et al., 2011). This is typically regarded to be a salmon-associated species (Williams et al., 1990), which is psychrotrophic and has been investigated with a view to its use as a biopreservative, albeit not in a dairy context (Fall et al., 2010). Little is known regarding its contribution to dairy products and how it has adapted to grow in dairy environments.

5.2 *Lactobacillus*

The genus *Lactobacillus* is very diverse and, according to the most recent estimations, consists of 174 different species and 27 subspecies (www.bacterio.cict.fr). Lactobacilli can be found in rich, carbohydrate-containing niches, including those associated with plants, animals, silage and raw milk (Bernardeau et al., 2008). An ever greater understanding of *Lactobacillus* biology has led to the use of strains of *Lactobacillus* for an increasing range of industrial dairy applications. In particular, their proteolytic activity and ability to produce aroma compounds and exopolysaccharides can contribute to the quality and nutritional value of dairy products (Leroy and De Vuyst, 2004). Lactobacilli that are of particular importance within the dairy industry are *Lactobacillus helveticus*, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Lactobacillus delbrueckii* ssp. *lactis* (the latter two species will be referred to as *L. bulgaricus* and *L. lactis* hereafter).

L. helveticus was first described by Orla-Jensen in 1919 as an isolate from an Emmental cheese (Naser et al., 2006) but it has since been evident that representatives of this species are commonly isolated from raw milk and

raw milk based products (Quigley et al., 2011). *L. helveticus* has a number of traits which are desirable with respect to cheese production. These include rapid autolysis of the strains, which results in the release of intracellular enzymes and a reduction in bitterness and increased flavour notes in cheese (Broadbent et al., 2011). *L. helveticus* is also characterised by its ability to grow at relatively high temperatures (55°C) (Hannon et al., 2003, Kiernan et al., 2000) and is the most proteolytic of the LAB frequently used in the manufacture of dairy products. The release of free fatty acids following lipolysis introduces important flavour compounds (Hickey et al., 2007). Genome sequencing of *L. helveticus* DPC4571, a Swiss cheese isolate, revealed that the presence of a high percentage of pseudogenes which are associated with loss-of-function events and presumably reflect adaptation to the dairy niche (Callanan et al., 2008). The growth of *L. helveticus* in milk is dependent on a complex system of proteolytic enzymes, which collectively enable strains to access essential amino acids (Christensen et al., 1999). Genome sequencing of DPC4571 revealed the presence of a number of peptidase genes (*pepE*, *pepQ*, *pepT* and *pepD*) that are likely involved in the proteolytic activity of this strain. PepQ is a proline-specific peptidase, which may be significant given that milk casein has a relatively high number of proline residues. Also, PepE, an endopeptidase, may have a role in reducing bitter defects during cheese ripening. DPC4571 lacks genes for colonisation and interaction with the mucosal surface that are present in related gastrointestinal probiotic species. These traits include surface proteins, cell-wall anchoring proteins and mucus binding proteins (Callanan et al., 2008). An isolate from naturally fermented milk, *L. helveticus* H10, has a larger genome than DPC4571 (Zhao et al., 2011). Most of the functional genes of this strain are conserved with DPC4571, but there are also 300 unique genes and 130 genes that are absent compared to DPC4571. *L. delbrueckii* can be divided into three major subspecies, *i.e.* ssp. *delbrueckii*, which is plant derived, ssp. *bulgaricus* and ssp. *lactis* (Giraffa et al., 2008). Representatives of the latter two subspecies have been regularly detected in raw milk samples, as well as being dominant populations in many traditionally manufactured cheeses and Protected Designation of Origin (PDO) cheeses (Morandi et al., 2011, Randazzo et al., 2002, Torriani et al., 1999). Both exhibit strong

proteolytic activity (Agyei and Danquah, 2012, Giraffa et al., 2004, Kholif et al., 2011). *L. bulgaricus* is, as a consequence of its worldwide use in yoghurt production, one of the most important dairy associated lactobacilli. It acts synergistically with *S. thermophilus*, allowing rapid growth and acidification with desired organoleptic properties (Herve-Jimenez et al., 2009). A number of factors are known to play a role in this 'protocooperation' process including degradation of milk proteins by *L. bulgaricus*; also the production of formate and CO_2 by *S. thermophilus* can stimulate the growth of *L. bulgaricus*. Genomics has revealed important factors which may play a role in this beneficial interaction. While *L. bulgaricus* encodes a full set of genes for biosynthesis of folate, it is thought that *S. thermophilus* is required to produce *p*-aminobenzoate, which feeds this pathway. Also, *in silico* analysis predicted the existence of a limited number of cell wall-bound and extracellular proteins which could contribute to direct contact between *L. bulgaricus* and *S. thermophilus* (van de Guchte et al., 2006). As with other milk-associated microorganisms, genomic studies suggest that the genome of *L. bulgaricus* is in an active state of gene elimination and size reduction but does contain genes encoding complete transport systems for lactose as well as mannose, glucose, fructose and glycerol (van de Guchte et al., 2006). As expected, strain-specific features are evident. For example, the industrially important bacterium *L. bulgaricus* strain 2038 has a number of unique features including a gene set involved in exopolysaccharide (EPS) synthesis, which may improve syneresis (separation of liquid) as well as texture, viscosity and mouth-feel of the final product. This strain also contains a larger genome than other strains (Hao et al., 2011).

There are several other lactobacilli in raw milk that increase in number during the manufacture of dairy products and can become particularly dominant during the ripening of cheese (Henri-Dubernet et al., 2008). These populations, which are often referred to as non-starter LAB (or NSLAB), have an ability to adapt to the conditions of cheese ripening, where many nutrients are depleted, pH is reduced and moisture content is low. Here, they are able to carry out proteolysis and lipolysis to produce many end-products that contribute to flavour and texture development of cheese (Smit et al., 2005). These include *L. casei, L. paracasei, L. plantarum/paraplantarum, L.*

rhamnosus and *L. curvatus*, *L. brevis, L. sake, L. pentosus*, *L. acidophilus, L. reuteri, L. johnsonii, L. crispatus, L. fermentum, L. buchneri* and *L. gasseri*. While the levels of these species can be under-estimated if culture-dependent methods are relied upon exclusively, the use of culture-independent techniques such as DGGE, TTGE and qPCR, or a combination of these with culturing, has addressed this issue in a number of instances.

5.3 *Streptococcus*

The genus *Streptococcus* consists of 97 species and 17 subspecies (www.bacterio.cict.fr). Although many genera of streptococci are pathogenic, *S. thermophilus* carries a 'generally regarded as safe' (GRAS) status (Facklam, 2002) and is frequently isolated from dairy environments, including raw milk, natural starter cultures and cheese curds (Duthoit et al., 2005, Randazzo et al., 2006, Santarelli et al., 2008). Strains of *S. thermophilus* have also been detected in the teats of cow's, cowsheds and dairy facilities (Braem et al., 2012, Vacheyrou et al., 2011). *S. thermophilus* is a thermophilic LAB widely used as a starter culture in the manufacture of dairy products. It is often regarded as the second most important industrial dairy starter after *L. lactis*. Its importance in dairy products is due to its ability to rapidly convert lactose to lactate, bringing about a rapid decrease in pH and the production of important metabolites including low levels of formate, acetoin, diacetyl, acetaldehyde and acetate (Ott et al., 2000). Many *S. thermophilus* strains produce exopolysaccharides (EPS) that contribute to the desirable viscous texture and rheological properties of fermented milk products, particularly yoghurt. In cheese, *S. thermophilus* is used alone or in combination with several lactobacilli and mesophilic starters, but in yoghurt it is always used with *L. bulgaricus*. While *S. thermophilus* is, in general, readily isolated by traditional microbiological methods, there are some instances where it has been overlooked and rapid molecular and culture-independent methods, such as SSCP (Randazzo et al., 2002) and DGGE (Duthoit et al., 2005), were required to detect its presence.

Whole genome sequencing of *S. thermophilus* has revealed that this bacterium exhibits 80% similarity with other streptococci, indicating that it shares a substantial part of its overall physiology and metabolism with its

pathogenic relatives. Analysis suggests that their common ancestor dates back 3,000 - 30,000 years, roughly corresponding to the origin of human dairy activity (*i.e.* ~7,000 years ago). The species lacks a number of functional genes typically found in other streptococci, including many genes involved in carbohydrate utilization as well as virulence-related genes, e.g. genes for some surface proteins that are required for adhesion to mucosal surfaces, and antibiotic modification genes. This lack provides strong evidence to support the GRAS status of *S. thermophilus*. Genome sequencing has also revealed the presence of more than 50 insertion sequences which have shaped the genome and, in part, are associated with genes involved in the adaptation of the species to milk. Indeed, a number of genes appear to have been acquired from other dairy microorganisms through horizontal gene transfer (Bolotin et al., 2004). *S. thermophilus* strain ND03, isolated from naturally fermented yak milk, contains unique genes not previously found in other strains. These are contained within a large insertion island and encode a transposase, a glutamate decarboxylase, an acetyltransferase, a polysaccharide biosynthesis protein and proteins associated with EPS biosynthesis (Sun et al., 2011). A comparative genome analysis of 47 *S. thermophilus* strains has revealed that the gene content can be split into core genes (58%; present in all strains) and non-core genes. The non-core genes can be split into conserved genes (14%), variable genes (20%) and acquired genes (8%). The latter genes - in addition to those referred to above with respect to ND03 - encode, for example, bacteriocins, efflux/uptake pumps, proteins involved in peptide metabolism, phage proteins as well as phage resistance genes (Rasmussen et al., 2008).

Other streptococci that have been associated with milk and milk products include *Streptococcus uberis*, *Streptococcus agalactiae, Streptococcus dysgalactiae Streptococcus bovis* and *Streptococcus macedonicus. S. macedonicus* has been isolated from artisanal raw milk cheeses (De Vuyst and Tsakalidou, 2008, Pacini et al., 2006) and has displayed some desirable characteristics from a dairy technology perspective. These include the ability to acidify, produce peptidases and the generation of inhibitory compounds while, importantly, lacking antibiotic resistance and haemolytic activity (Lombardi et al., 2004, De Vuyst and Tsakalidou, 2008).

Genome sequencing of a dairy isolate of *S. macedonicus* revealed that the bacterium is undergoing regular genome decay as indicated by the presence of a large number of pseudogenes. The genus also appears to lack the *pil1* locus that is involved in instances of infectious endocarditis caused by pathogenic relatives (Papadimitriou et al., 2012). Nonetheless, further investigations are required to definitively establish the safety status of the species with respect to its use in dairy products. An African dairy isolate, *Streptococcus infantarius* ssp. *infantarius* strain CJ18, contains plasmids with high sequence identity to *L. lactis* sequences. The presence of a *gal-lac* operon suggests an evolutionary adaptation to the dairy niche (Jans et al., 2012). More recently, comparative genomic analysis has revealed that the *gal-lac* operon of CJ18 has 91% identity with that of *S. thermophilus*. CJ18 also contains an oligopeptide transport operon, which is important during growth in milk for the uptake of peptides and amino acids, and lacks classical streptococcal virulence factors (Jans et al., 2013).

Many of the other streptococci regularly detected in bovine milk are associated with mastitis infection. Mastitis-associated pathogens typically infect the teat canal of cow's and pass into the milk during milking. The presence of these microorganisms impairs milk quality and the quality of subsequent products (Barbano et al., 2006). *S. uberis* is an animal pathogen and one of the major causes of bovine mastitis worldwide (Bradley et al., 2007). Genomic analysis provides evidence of the nutritional flexibility of *S. uberis* that allows it to occupy various ecological niches, including mammary glands (Ward et al., 2009). *S. dysgalactiae*, a contagious and environmental pathogen, also accounts for a notable proportion of bovine mastitis infections (Todhunter et al., 1995). A number of genes are involved in its ability to adhere to the mammary gland, where it can survive and is protected from the immune system as well as therapeutics. The severity of disease differs with genotype (Beecher et al., 2012). It has been suggested that *S. dysgalactiae* present in the mammary gland degrades the proteose peptones of milk prior to milking and consequently results in a reduced ability of milk to coagulate during fermentations (Merin et al., 2008). Similarly, *S. agalactiae* is a major causative agent associated with mastitis in cows. It has been proposed that a number of genes, including a *lac* operon, have been acquired through lateral

gene transfer to allow this bacterium to adapt to the bovine host. An unusually high number of insertion elements have also been detected suggesting frequent genomic rearrangement (Richards et al., 2011). Finally, *S. bovis* is an opportunistic human pathogen, which is often associated with infections in immune-compromised or cancer patients. It can also be detected in other environments including fermented foods (Barile et al., 2012).

5.4 *Propionibacterium*

The genus *Propionibacterium* comprises two distinct groups from different habitats, *i.e.* strains typically found on human skin, referred to as the "acnes group", and strains isolated from milk and dairy products, referred to as 'dairy' or 'classical' propionibacteria (Meile et al., 2008). Notably, it has also been claimed that dairy propionibacteria possess health-promoting characteristics (Cousin et al., 2011). The dairy group of propionibacteria comprises four species, *Propionibacterium freudenreichii, Propionibacterium acidipropionici, Propionibacterium jensenii* and *Propionibacterium thoenii* (www.bacterio.cict.fr). *P. freudenreichii* serves as a starter in Swiss-style cheeses. It was first isolated over a century ago from an Emmental cheese and contributes to hole or "eye" formation and flavour formation in these cheeses (Langsrud and Reinbold, 1973). The other species of dairy *Propionibacterium* are usually isolated from milk and different cheese types (Meile et al., 2008). The characteristic trait of *P. freudenreichii* is the fermentation of lactate into propionate, acetate and CO_2, while associated distinctive flavours arise from the formation of fatty acids, through lipolysis, and of branched-chain acids from the catabolism of amino acids. Due to its long documented use in cheese manufacture, *P. freudenreichii* has a GRAS status (Cousin et al., 2011). Whole genome sequencing of *P. freudenreichii* CIRM-BIA1^T revealed its ability to cope with different stresses including oxidative, bile salt and temperature stresses, an ability to resist phage attack, to accumulate nutrients and mobilise these during periods of starvation and to synthesise most vitamins and amino acids. Sequencing also revealed the presence of a number of genes that are thought to encode surface proteins potentially involved in adhesion and immunoregulatory activity. The ability of *P. freudenreichii* to utilise lactose has been found to be strain dependent.

CIRM-BIA1^T possesses a lactose utilisation locus encoding a β-galactosidase, a galactose transporter and an UDP-glucose isomerise. This locus is surrounded by transposable elements, is highly similar to corresponding regions in strains of *Clostridium* and *Mannheimia* and, thus, is believed to have been acquired through gene transfer to facilitate the adaptation of the microorganism to the dairy environment. Importantly, genes involved in pathogenicity of *P. acnes* are absent from CIRM-BIA1^T (Falentin et al., 2010).

5.5 *Leuconostoc*

The genus *Leuconostoc* consists of 23 species and 4 subspecies (www.bacterio.cict.fr). *Leuconostoc* spp. are frequently associated with plant material but some, and in particular the species *mesenteroides* and *pseudomesenteroides*, are also found in milk. However, it is possible that this is due to their introduction during the collection of milk or subsequent storage and processing. Notably in this regard, *Leuconostoc* spp. have the ability to survive on surfaces, tools and pasteurisers for long periods of time and to resist heat treatments and refrigeration temperatures (Hemme and Foucaud-Scheunemann, 2004). *Leuconostoc* spp. grow poorly in milk due to a lack of sufficient proteolytic activity and thus require the addition, or generation by other microorganisms, of amino acids or peptides to stimulate growth (Hemme and Foucaud-Scheunemann, 2004, Vedamuthu, 1994). *Leuconostoc* spp. have the ability to produce gas (CO_2), which is responsible for eye formation in some artisanal raw milk or blue veined cheeses (Cardamone et al., 2011), metabolise lactose and citrate, and produce lactate, acetate, ethanol, acetaldehyde, diacetyl, acetoin and 2,3-butanediol, which contribute to the organoleptic properties of fermented dairy products (Sanchez et al., 2005, Vedamuthu, 1994). Due to these attributes, *Leuconostoc* spp. can act as beneficial NSLAB cultures. Genome sequencing of the dairy isolate *Leuc. pseudomesenteroides* strain 4882 has further highlighted the beneficial attributes, e.g. genes involved in carbohydrate fermentation, protein and amino acid metabolism, and a key pathway in production of aromatic compounds from citrate (Meslier et al., 2012b). Notably, while phenotypic assays do not always reliably differentiate between species or subspecies of *Leuconostoc*, molecular methods can facilitate the rapid characterisation of

Leuconostoc to species level (Duthoit et al., 2003, Martin-Platero et al., 2009, Sanchez et al., 2006).

5.6 *Enterococcus*

Enterococci are the most controversial group of food-associated LAB. Enterococci occupy a diverse range of ecological niches that include the gastrointestinal tracts of humans and animals (Giraffa, 2002) and, depending on the strain in question, can be considered to be starter cultures, probiotics, spoilage or pathogenic organisms (Bhardwaj et al., 2009). Due to their psychrotrophic nature, ability to survive adverse conditions, including high temperature and high salinity environments, and adaptability to different growth substrates and growth conditions, enterococci can survive refrigeration. In laboratory based experiments, strains of this bacterium have been shown to potentially survive pasteurisation and thus may be part of the microbial populations in both raw and pasteurised milk as well as in subsequent products (Giraffa, 2003, Ladero et al., 2011, McAuley et al., 2012). Studies on raw milk cheeses indicate that enterococci are a common, and frequently important, component of the natural cultures involved in fermentations and contribute to ripening, taste and flavour (Foulquié Moreno et al., 2006). The most common enterococcal species in milk and dairy products are *Enterococcus faecalis* and *Enterococcus faecium* but others, including *Enterococcus durans* (Franciosi et al., 2009), *Enterococcus italicus* and *Enterococcus mundtii*, are also encountered. Enterococci contribute to fermentations due to their proteolytic activity, ability to hydrolyse milk fat and contribution to the development of flavour compounds, including acetaldehyde, acetoin and diacetyl (Franz et al., 1999). Recent genome sequencing projects identified large gene sets related to dairy adaption, including genes involved in lactose and galacto-oligosaccharide utilization, in *Ent. mundtii*. Furthermore, a large number of putative antibiotic resistance determinants have also been found (Magni et al., 2012). Importantly, food isolates of *Ent. faecalis* lack a large number of genes which are present in clinical isolates. These traits (including genes for adhesion and an entire prophage) are believed to contribute to the development of human infection (Lepage et al., 2006).

6. Other milk microorganisms with potential technological relevance

There are some other groups of microorganisms of technological relevance, albeit not regarded as being as important as those discussed above, that are present in low quantities in raw milk. These include a number of bacteria, including Gram-positive and -negative bacteria, as well as yeast and mould populations.

6.1 Gram-positive subpopulations

Corynebacterium spp. were detected in milk over 40 years ago (Jayne-Williams and Skerman, 1966) and have also been found on the teat surface and throughout the farm environment (Braem et al., 2012, Vacheyrou et al., 2011). Coryneform bacteria are generally regarded as being important components of the surface of smear-ripened cheese but can also be located in cheese cores (Duthoit et al., 2003). These bacteria can contribute to cheese flavour and aroma due to their ability to produce volatile sulfur compounds giving notes of garlic, onion and even cabbage to the cheese. These compounds result from the production of methanethiol, sulfides, thiols and thioesters (Bloes Breton and Bergere, 1997). Sequencing of *C. casei* UCMA 3821, *C. variable* DSM 44702 and *C. bovis* DSM 20583 has revealed genes involved in iron acquisition and uptake, an important feature of cheese surface bacteria. Also present are genes involved in utilisation of alternative carbon and sulfur sources, amino acid metabolism and fatty acid degradation. The genetic repertoire of these strains also highlights their ability to catabolise lactate and propionate, utilise external caseins and produce acetoin, butanediol and methanethiol, which are important with respect to the flavour of smear-ripened cheeses (Monnet et al., 2010, Schröder et al., 2012, Schröder et al., 2011).

Arthrobacter spp. are commonly isolated from raw milk (Masoud et al., 2012, Verdier-Metz et al., 2009) and are thought to enter from the dairy facility as well as the teat surface (Vacheyrou et al., 2011). While little is known with respect to the influence of these bacteria on cheese development, they are an important microbe on the surface of smear-ripened cheese where they

contribute to colour, flavour and textural development. *Arthrobacter arilaitensis* is perhaps the most important cheese-associated species and genome sequencing of strain Re117 reveals the presence of several genes which reflect its adaptation to growth in dairy/cheese environments including salt tolerance, galactose metabolism and enzymes for catabolism of fatty acids, amino acids and lactate. As with other smear-associated bacteria, *Arthrobacter* spp. possess a gene set involved in iron transport (Monnet et al., 2010). Similarly, *Brevibacterium*, which is commonly detected in raw milk (Desmasures and Gueguen, 1997, Lafarge et al., 2004, Masoud et al., 2012, Raats et al., 2011), is known for its association with characteristic taste, aroma and colour of smear-ripened cheeses. *Brevibacterium linens* is particularly important in this regard (Irlinger and Mounier, 2009). Another genus detected in raw milk, *Carnobacterium*, consists of 11 species (www.bacterio.cict.fr) (Cailliez-Grimal et al., 2005). The *Carnobacterium* species most frequently isolated from dairy environments is *Carnobacterium maltaromaticum*. *C. maltaromaticum* was first isolated from milk in 1974 and was originally named *Lactobacillus maltaromaticus* (Mora et al., 2003). *Carnobacteria* are slow acidifiers and therefore are not suitable for use as starter cultures but can be considered to be beneficial NSLAB due to their aromatic and flavour contributing end-products (Afzal et al., 2010). These include malty aromas as well as alcohol and fruity odours in cheese, though some strains have been linked with sweat, faecal and rotten-fruit associated flavours (Marilley and Casey, 2004). The occurrence of *Carnobacterium* in dairy products is probably underreported due to the frequent use of acetate containing media, such as MRS medium, as acetate inhibits the growth of these microbes (Leisner et al., 2007).

Bifidobacterium represents an important genus, which is generally regarded as health promoting and is most commonly associated with the gastrointestinal tract of humans and animals (Lamendella et al., 2008). It is also frequently detected in raw milk and fermented dairy products (Delcenserie et al., 2005) despite the fact that many *Bifidobacterium* strains have stringent nutrient requirements and are generally thought to grow poorly outside of the gut (Lamendella et al., 2008). In dairy products, the presence of bifidobacteria results in increased levels of lactate and acetate but does not

influence sensory or textural properties (Dinakar and Mistry, 1994). Finally, the significance of coagulase-negative staphylococci (CNS) with respect to dairy fermentations has been the subject of much debate. The species typically isolated from milk include *S. equorum*, *S. xylosus* and *S. carnosus*. These bacteria are salt- and acid-tolerant. Although no CNS of dairy origin have been associated with food poisoning or human pathology, a few cases of nosocomial infection caused by *S. caprae*, *S. capitis* or *S. sciuri* have been reported in patients with depressed immune systems (Irlinger, 2008). A recent study of isolates from milk and cheese revealed 17 CNS species. Ten of these contained transferable antibiotic resistance genes and one third exhibited haemolytic activity (Ruaro et al., 2012), indicating that the safety of these bacteria must continue to be assessed.

6.2 Gram-negative subpopulations

Gram-negative bacteria are very common in dairy foods. They can reach high levels (10^6-10^7 cfu g^{-1}) in cheeses and usually consist of a diverse number of species. Although gram-negative bacteria are regularly considered as indicators of poor hygiene and may constitute a health risk if pathogenic species are present, some may play roles in dairy fermentations by contributing positively or negatively to the sensory quality of dairy products (Delbès-Paus et al., 2011, Delbès-Paus et al., 2012). These issues are addressed in greater depth in this section.

The presence of high numbers of Gram-negative bacteria in milk has been noted in situations where hygiene standards are low and generally reflect poor udder preparation, poor sanitation or deficiencies with respect to the hygiene of equipment. In one instance, milk sampled at the farm, at milk collection and milk transportation was found to be contaminated with *E. coli* (29.6%), *P. aeruginosa* (18.5%) and *Klebsiella pneumoniae* (16.7%) and, to a lesser extent, *Enterobacter aerogenes*, *Alcaligenes faecalis*, *Proteus mirabilis* and *Citrobacter freundi*. It was noted that no Gram-negative bacteria were isolated from pasteurised milk samples (Garedew et al., 2012). In a study investigating the presence of Gram-negative bacteria in different cheeses produced in France, 173 isolates were isolated. Nearly half of all isolates were representatives of *Enterobacteriaceae*. Overall, 26 different genera were

present. The most frequent isolates included *Proteus*, *Psychrobacter*, *Halomonas*, *Serratia* and *Pseudomonas* representing almost 54% of the total isolates. Milk and cheese core samples also contained *Chryseobacterium*, *Enterobacter* and *Stenotrophomonas* while surface samples were dominated by *Proteus*, *Psychrobacter*, *Halomonas* and *Serratia (Coton et al., 2012)*. When a model cheese system was employed to assess the consequences of the presence of some of these Gram-negative bacteria, it was established that the majority had little influence on colour, odour and volatile compounds (Delbès-Paus et al., 2011). However, *Hafnia alvei* did contribute to the production of volatile compounds, and of volatile sulfur compounds in particular. Furthermore, *Psychrobacter celer* was found to flourish within the cheese surface smear during ripening, contributing to the production of volatile compounds such as aldehydes, ketones and sulfur compounds (Delbès-Paus et al., 2011, Irlinger et al., 2012). Another Gram-negative species, *Proteus vulgarius* M10, has been shown to produce high concentrations of flavour compounds, particularly branched-chain alcohols during ripening (Deetae et al., 2009). These studies reveal the high biodiversity of Gram-negative bacteria among raw milk and dairy products and suggest that they may play a role in dairy fermentations. However, the fact that one of the studies referred to above revealed that ~50% of the Gram-negative strains isolated were resistant to several antibiotics is a particular cause for concern (Delbès-Paus et al., 2011). Given this observation, and the association between particular Gram-negative bacteria with milk spoilage or disease, the presence of Gram-negative bacteria in these products will in general continue to be regarded as undesirable.

6.3 Fungal populations

Yeasts and moulds can also be important microbial populations within raw milk. The fungal composition of raw milk can be influenced by the physiological state of the animal, as well as the weather, feeding and season (Callon et al., 2007, Vacheyrou et al., 2011). As with bacteria, the extent of the fungal population in raw milk and dairy products is often underestimated. However, the development of culture-independent DNA-based methods, such as those targeting the internal transcribed spacer (ITS) region of fungi, has

addressed this issue (Alessandria et al., 2010, Callon et al., 2006). This ITS region of fungi is particularly useful because of its high copy number, phylogenetic utility and the availability of universal primers to generate PCR amplicons. This and other culture-independent approaches, such as denaturing high-performance liquid chromatography, have led to the detection of fungi, e.g. *Torrubiella* and *Malassezia*, which had not previously been detected in milk (Delavenne et al., 2011). While a relatively small number of yeast species occur in raw milk, they are persistent and can be detected at relatively high levels, *i.e.* 10^2-10^4 cfu ml^{-1} (Lagneau et al., 1996). Yeasts species that have been detected in raw milk include *Kluyveromyces marxianus, Kluyveromyces lactis, Rhodotorula mucilaginosa, Debaryomyces hansenii, Geotrichum candidum, Geotrichum catenulate, Pichia fermentans, Candida sake, Candida parapsilosis, Candida inconspicua, Trichosporon cutaneum, Trichosporon lactis, Cryptococcus curvatus, Cryptococcus carnescens* and *Cryptococcus victoriae* (Delavenne et al., 2011). Yeasts can play a major role in dairy fermentations due to a number of their physiological and biochemical characteristics, including the ability to utilise lactose or galactose, e.g. in *D. hansenii* (Van den Tempel and Jakobsen, 2000), high proteolytic or lipolytic activity e.g. in *Yarrowia lipolytica, G. candidum* (Sacristán et al., 2012), and the ability to grow at low temperatures and to tolerate high salt concentrations. In cheese, yeasts secrete enzymes that play a key role in texture and produce various aromas during ripening. *K. marianus* is of particular interest due to its fast growth rate, thermotolerance, the ability to assimilate a wide range of sugars, the secretion of lytic enzymes and the production of ethanol by fermentation (Lane and Morrissey, 2010). Moulds are typically present at lower levels than yeasts (Arora et al., 1991). Moulds have the ability to enhance the flavour and aroma and modify the texture and structure of milk-derived products as a consequence of bringing about extensive proteolysis and lipolysis. The mould genera that are most commonly detected in raw milk include *Penicillium, Geotrichum, Aspergillus, Mucor* and *Fusarium* (Lavoie et al., 2012). At the species level *Fusarium merismoides, Penicillium globrum, Penicillium roqueforti, Aspergillus fumigatus, Engyodontium album* as well as species of *Cladosporium* and *Torrubiella* are common (Delavenne et al., 2011).

In recent years, genome sequencing has been particularly useful with respect to enhancing our understanding of milk-borne microorganisms by, for example, highlighting the phenomenon of genomic decay and adaptation to the milk environment, revealing potential sources of evolutionary origin and identifying genes that contribute to flavour development in dairy products. This information can allow industry to develop novel starter cultures, determine how best to enhance the efficacy of existing strains and provide re-assurances in terms of the 'GRAS' status of some of these technologically important microorganisms.

7. Impact of storage conditions and downstream treatments on the microbiology of raw milk

7.1 Cold Storage

It is important to understand the changes which can occur in the microbiology of raw milk during its storage and as a consequence of subsequent treatments. Milk is typically stored at refrigeration temperatures that reduce the growth of most bacteria with the exception of psychrotolerant microorganisms that can proliferate under these conditions and become a major cause of milk spoilage (Eddy, 1960, Morita, 1975, De Jonghe et al., 2011). This is primarily a consequence of the production of extracellular enzymes, with lipases and proteases being most important. These lipases degrade milk fat causing rancidity, while proteases degrade casein producing a gray colour and bitter off-flavours (De Jonghe et al., 2011). Investigations of seasonal variations of microbial growth in raw milk have, unsurprisingly, established that psychrotolerant bacteria exhibit better growth and protease production in winter milk rather than in summer milk (Marchand et al., 2008). *Pseudomonas* spp., which are commonly found in raw milk, are the most common cause of milk spoilage (Ercolini et al., 2009). The *Pseudomonas* species most commonly detected in milk and cheeses are *Pseudomonas fluorescens, Pseudomonas gessardi, Pseudomonas fragi* and *Pseudomonas lundensis* (Mallet et al., 2012). These bacteria can become the predominant microorganisms in raw milk stored at low temperatures, constituting up to 70 - 90% of the microbial population (Sorhaug and Stepaniak, 1997). Many other

psychrotolerant microorganisms are present in milk but are generally less important than *Pseudomonas* with respect to milk spoilage. In one study these were identified as being strains of *Acinetobacter, Microbacterium, Aeromonas, Enterobacter, Flavobacterium, Corynebacterium, Clostridium, Bacillus, Staphylococcus* and some LAB (Hantsis-Zacharov and Halpern, 2007). Another study, assessing the overall impact of refrigeration (24 h) on the microbial content of raw milk, particularly noted increases in the number of *Listeria innocua, Listeria monocytogenes, L. fermentum, S. epidermidis, P. fluorescens, Ent. faecium, Ent. hirae, Ent. durans, Leuc. carnosum, S. dysgalactiae, Hafnia alvei, Serratia marcescens, Klebsiella pneumoniae, Kocuria rosea,* Propionic Acid Bacteria and *Aeromonas* (Lafarge et al., 2004). Notably, some of the latter would be typically regarded as thermophilic microorganisms. A similar study, but carried out over a 48 h period, specifically highlighted increases in *Pseudomonas* and *Acinetobacter* spp. (Raats et al., 2011). Newly identified psychrotrophs, such as *Chryseobacterium* (Hantsis-Zacharov and Halpern, 2007, Hantsis-Zacharov et al., 2008a, Hantsis-Zacharov et al., 2008b) and *Epilithonimonas* spp. (Shakēd et al., 2009), have also been detected in raw milk. However, their involvement in milk spoilage is unclear. A number of psychrotrophic spore-forming species have also been identified and will be discussed in the next section. Yeasts and moulds have also been associated with milk spoilage (Agarwal et al., 2012).

7.2 Pasteurisation

Pasteurisation of raw milk is carried out to reduce the microbial load of milk and, in particular, to limit the number of spoilage microbes and to prevent food-borne disease. However, this process also reduces the number of microbes that would typically contribute to desirable sensory properties associated with raw milk cheeses. In these instances starter cultures that are known to generate desirable flavours and aromas, as discussed above, are added to the milk post-pasteurisation. The typical milk pasteurisation treatment is a ‘high temperature short time’ (HTST) approach involving heating to 72°C for 15 s. Some countries have increased the exposure temperature and/or time (Martin et al., 2011). While this can help to further

reduce bacterial counts (Fromm and Boor, 2004) and to eliminate microbes of concern including *Mycobacterium avium* ssp. *paratuberculosis* (MAP) (Grant et al., 2002) and *Listeria monocytogenes* (Doyle et al., 1987), there have also been some suggestions that this approach can encourage the activation of spores which may be dormant in milk (Ranieri et al., 2009). The heat treatment of milk typically reduces psychrotrophic and mesophilic populations leaving two main groups to consider thereafter, *i.e.* thermoduric microorganisms (discussed below) and bacteria introduced through post-pasteurisation contamination. Following pasteurisation some microorganisms may enter into a 'viable-but-non-culturable' (VBNC) state, meaning that they may be underestimated by traditional culture methods (Bartoszcze, 2009). The findings of a recent culture-independent study conducted by our group are consistent with this theory, revealing a more diverse bacterial population in pasteurised milk than expected (Quigley et al., 2013).

When one considers thermoduric bacteria, it is particularly important to keep the issue of spore-forming microorganisms in mind. These bacteria may enter the milk chain from soil, silage and bedding material and, significantly, are resistant to pasteurisation. Spore-formers such as *Clostridium sporogenes*, *C. butyricum* and *C. tyrobutyricum* have the potential to survive and grow at refrigeration temperature, as well as the potential to utilise carbohydrates, proteins and lactate from milk (Driehuis, 2013). Indeed, clostridia have been identified in raw milk quite frequently (Cremonesi et al., 2012, Herman et al., 1995, Lopez-Enriquez et al., 2007) and can contribute to the spoilage of subsequent cheese products by causing a late blowing defect, which is particularly associated with *C. tyrobutyricum*, leading to off-flavours and textural defects in cheese (Cocolin et al., 2004, Le Bourhis et al., 2005). Culture-independent DGGE/TTGE has identified *Clostridium tyrobutyricum*, but also *C. sporogenes, C. butyricum* and *Clostridium beijerinckii* as possible causes. Other spore-forming contaminants of milk include *Bacillus cereus*, *Bacillus sporothermodurans* and *Geobacillus stearothermophilus*. *B. cereus* is a major spoilage organism of pasteurised milk and milk products stored at refrigeration temperature, causing off-flavour and curdling. This bacterium is also a concern for food safety as it can produce different types of toxins and is

a potential food poisoning agent (Driehuis, 2013). In the EU in 2010, 3.8% of all milk samples tested were positive for *Bacillus* toxin (EFSA, 2012).

7.3 Bacteriophage

Lytic bacteriophages are other spoilage agents that are naturally present in raw milk. These viruses infect bacteria and, after intracellular replication, lyse their host cells (Marcó et al., 2012). Phages can survive at low temperature, resist a variety of different treatments and negatively affect the quality, safety and value of dairy products (cheese in particular). Phages which target important starter or adjunct species, such as *L. lactis, S. thermophilus, L. helveticus* and *L. delbrueckii*, have long been associated with causing a delay or disruption of fermentation processes, resulting in slow acidification, undesirable organoleptic properties or complete loss of batches (Emond and Moineay, 2007). Raw milk is the most prominent source of phages within the dairy environment, with concentrations ranging from 10^1 to 10^4 phage ml^{-1} (Madera et al., 2004). Phages can also gain access to the dairy environment by aerosols, personnel, equipment, work surfaces and dairy by-products (Verreault et al., 2011). Thus, attaining a phage-free environment is not a realistic goal. While the phage concentration is higher in raw milk products, it has also been reported that many dairy phages are able to survive the pasteurisation of milk (Suarez and Reinheimer, 2002, Abedon, 2009). Due to the severe economic loss that phages can cause constant monitoring of the environment is required. Traditionally, standard microbiological methods were employed. However, molecular methods based on PCR and quantitative PCR allow the rapid detection and classification of phages from different dairy matrices (Binetti et al., 2008). While many dairy bacteria can be infected by phage, there are a number of industrially important strains that have inherent resistance to phage. Genome sequencing studies have revealed that a number of strains, such as *L. helveticus* DPC4571, *P. freudenreichii* CIRM-BIA1^T and some *S. thermophilus* strains to name but a few, possess features to help these bacteria withstand phage attack.

7.4 Biopreservative potential of raw milk microorganisms

The production of an antimicrobial can be regarded as a beneficial probiotic trait. The diverse populations in raw milk produce many antimicrobials including bacteriocins, antifungals, organic acids and hydrogen peroxide. As noted above, raw milk also contains phages. These might also be regarded as biopreservative agents that could be used to extend the shelf life and safety of fermented and other foods (Stiles, 1996).

Bacteriocins are antimicrobial peptides or proteins produced by bacteria and are typically active against closely related species, but can exhibit activity across broad genera. Bacteriocin producers are naturally immune to their own bacteriocins (Cotter et al., 2005). The most recent classification groups LAB bacteriocins into two classes; class I are post-translationally modified bacteriocins and class II are unmodified bacteriocins (Rea et al., 2011). Many LAB isolated from raw milk produce putative bacteriocin-like compounds and exhibit activity against *L*. *monocytogenes*, *S. aureus, C. tyrobutyricum, C. sporogenes, Ent. faecalis, Ent. faecium* and *Ent. durans* (Alegría et al., 2010, Ortolani et al., 2010, Perin et al., 2012). Some potent raw milk-derived bacteriocins have been characterised in depth. *L. lactis* strains produce the best characterized bacteriocin, nisin. Nisin is well-known due to its use in biopreservation throughout the world because of its wide spectrum of activity (Delves-Brougthon, 1990). *L. lactis* strains isolated from raw milk and raw milk products are capable of producing nisin with activity against *L. monocytogenes* as well as other pathogens including *E. coli* and *Staphylococcus* spp. (Alegría et al., 2010, Bravo et al., 2009, Cosentino et al., 2012, Ortolani et al., 2010, Perin et al., 2012). A huge variety of other bacteriocins are produced by *L. garvieae* (Florez et al., 2012, Villani et al., 2001), *S. thermophilus* (Gul et al., 2012), *S. macedonicus* (Georgalaki et al., 2002, Georgalaki et al., 2013), enterococci and *Leuconostoc* spp. (Izquierdo et al., 2009, Achemchem et al., 2006, Casaus et al., 1997, Giraffa and Carminati, 1997, Mirhosseini et al., 2010, Mathieu et al., 1993) and are active against many spoilage and pathogenic microorganisms. In addition to contributing to the control of pathogens and spoilage microbes in raw milk and resultant products, these bacteriocins can also be employed, through the

addition of producing LAB, fermentates or semi-purified preservatives, to enhance the safety of other foods (Cotter et al., 2005, Deegan et al., 2006).

Raw milk LAB isolates also produce organic acids, hydrogen peroxide and diacetyl (Stiles, 1996). These compounds can inhibit many potential pathogens and food spoilage microorganisms (Batdorj et al., 2007). Some strains of *L. acidophilus, L. casei, L. helveticus, L. bulgaricus, S. thermophilus* and *L. lactis* are also able to transform hippuric acid, which is naturally present in milk, into benzoic acid, thereby providing another natural preservative in milk products (Garmeine et al., 2010). Finally, common food-spoiling moulds and, to a lesser extent, yeasts can be inhibited by LAB metabolites from strains of *L. casei, L. reuteri, L. plantarum* and *L. buchneri* and strains of dairy propionibacteria (Delavenne et al., 2011, Voulgari et al., 2010, Lind et al., 2005, Lind et al., 2007).

8. Human health associations

8.1 Pathogenic bacteria associated with raw milk

Milk and dairy products are important staples of a healthy diet. However, if pathogenic microorganisms are not removed by pasteurisation, consumption of these products can represent a serious health risk. As mentioned above, these pathogens can originate from the mammary gland or associated lymph nodes of cow's suffering from systemic diseases or infections (Hunt et al., 2012, Oliver and Murinda, 2011) or from equipment, raw milk tankers and personnel (Giacometti et al., 2012, Teh et al., 2011, Rosengren et al., 2010). Ingestion of these microbes can lead to illnesses of varying severity. Typical symptoms can include fever, nausea, vomiting, diarrhoea and abdominal pains; in extreme cases, deaths can occur (Langer et al., 2012). Indeed, food poisoning from consumption of raw milk and such products over a period of 13 years (1993-2006) in the United States resulted in 1,571 reported incidences with 202 hospitalisations and 2 deaths. The main cause of illness was consumption of raw milk products contaminated with *Salmonella* spp., *Listeria* spp., *E. coli*, *Campylobacter* spp., *Brucella* spp. or *Shigella* spp. (Langer et al., 2012). A recent review, which examined multiple reports of milk-borne pathogen detection in bulk-tanks throughout different countries,

found that the percentage of tanks containing the different pathogens varied greatly. The occurrence of *Salmonella* ranged between 0 and 11.8% of milk samples in the USA/Canada and between 1.4 and 4% of samples in Asia, while the percentage of milk samples that was positive for the presence of *Listeria* ranged from 0 to 7% in USA/Canada and from 0 to 1.9% in Asia. *Campylobacter jejuni* was detected at 2 and 9.2% of milk samples from the USA, while the percentage of bulk tank milk worldwide that was positive for shiga-toxin producing *E. coli* varied from 0 to 33.5%. *Mycobacterium avium* ssp. *paratuberculosis* was present in European samples at frequencies of 1.6 to 19.7% and in Asia at 8.6 to 23%. *Brucella* was present in milk in Africa at 0 to 10%, increasing with increasing farming intensity (Oliver and Murinda, 2011).

Regardless of the frequency at which they are present, these pathogens can impact, in some instances severely, on health. *S. aureus* can be transferred to milk through the teat canal, equipment, the environment or human handling (Rosengren et al., 2010) and cause illness through the production of heat-stable enterotoxins, which can withstand pasteurisation (Balaban and Rasooly, 2000). Staphylococcal toxins were detected in 18.4% of cheeses assessed across the EU in 2010 (EFSA, 2012). *Coxiella burnetti,* the causative agent of Q fever can infect many animal species and it is thought that its association with cow's, sheep's and goat's is the main source of human infection. The infection may be acute, presenting flu-like symptoms which are self-limiting, or chronic, leading to endocarditis and hepatitis (Maurin and Raoult, 1999). *C. burnetti* is shed by the animal host through birth products, vaginal mucus, semen, faeces, urine and milk (Guatteo et al., 2006). While this bacterium can persist in dairy cattle populations (Astobiza et al., 2012, Tilburg et al., 2012), the consumption of raw or insufficiently pasteurised milk is rarely identified as a source of Q fever (Guatteo et al., 2006). Another zoonotic bacterium of health concern is *Mycobacterium bovis*. This bacterium causes the disease bovine tuberculosis in animals, with symptoms including fever, weakness, emaciation, inappetence and respiratory distress, and can lead to severe economic loss yearly (Thoen et al., 2006). This pathogen can also spread to humans through the ingestion of raw milk causing zoonotic tuberculosis, which is indistinguishable from human

tuberculosis (Thoen et al., 2006). Typically this concern is removed with pasteurisation, but remains a problem in instances where raw milk is still consumed daily (Coker et al., 2006, De la Rua-Domenech, 2006). *Mycobacterium avium* ssp. *paratuberculosis* (MAP) is the causative agent of paratuberculosis or Johne's disease, which primarily infects domestic animals. MAP survives and multiplies in the intestinal tract mucosa, where it causes both a decrease in the absorption of nutrients and chronic diarrhoea with consequent "wasting-away" of the animals. Animals may harbour this bacterium for a long period before symptoms arise making them dangerous vectors of infection. MAP can be shed into the external environment in animal feces or milk. Recently there has been increased concern with the association of MAP and Crohn's disease in humans, an inflammatory bowel disease, whose symptoms include abdominal pain, diarrhea, vomiting and weight loss. High prevalence of MAP has been reported in raw milk in developed countries (Argentina 8.3%, Czech Republic 2%, Ireland 0.3%, UK 6.9%, USA 0 - 28.6%) (Slana et al., 2008). Current commercial pasteurisation standards may reduce the number of viable MAP, but do not ensure destruction (Gao et al., 2002). However, the link between Crohn's disease and MAP still remains controversial and unclear (Chiodini et al., 2012). Another, relatively new, pathogen of concern to the dairy industry is Shiga-toxin producing *E. coli* (STEC). Although cow's are the main reservoir for STEC, other domestic animals including goat's and sheep's can also harbour this bacterium in their gastrointestinal tracts without any symptoms of disease and shed them from their faeces. If hygiene standards are not sufficiently high, milk can become contaminated during milking or processing. There are nine virulence genes associated with STEC strains. Two toxin genes, *stx1* and *stx2*, appear to be associated with bovine dairy products while *stx1c* and *stx2b* are more frequently associated with strains from sheep's and goat milk (Martin and Beutin, 2011).

Of the milk-borne pathogens, *L. monocytogenes, Yersinia enterocolitica* and *Brucella* spp. are a particular cause for concern as they are able to survive and multiply at refrigeration temperatures and may cause severe diseases. *Y. enterocolitica* is a major cause of acute gastroenteritis (Schiemann and Toma, 1978). The symptoms of illness can include diarrhea,

abdominal pain and fever and may mimic appendicitis, occasionally leading to misdiagnosis (Ackers et al., 2000). Although pasteurisation will kill *Y. enterocolitica*, if insufficient pasteurisation or re-contamination occurs, the bacterium can multiply under refrigeration temperatures (Schiemann and Toma, 1978). Similarly, during dairy product manufacture, where hygiene standards are poor, *Y. enterocolitica* can become prevalent (Harakeh et al., 2012). In 2010, *Yersinia* incidence in raw milk and low heat-treated milk products was low, with only 2 positive results reported within the EU (EFSA, 2012). *L. monocytogenes*, a common environmental isolate, causes the human disease listeriosis, which targets highly susceptible individuals, including pregnant, immuno-compromised or elderly people, and has a high fatality rate. Healthy adults are typically not at risk although they may experience flu-like or gastrointestinal symptoms (Liatsos et al., 2012). There is evidence to suggest that raw milk purchased from retailers represents a greater risk of *Listeria*-associated illness than milk obtained directly from milk tanks on farms, most likely as a consequence of the growth of the pathogen over the extended storage period (Latorre et al., 2011). *Brucella* spp. primarily cause disease in animals and from there are thought to enter into the milk supply. On consumption, these pathogens can provoke brucellosis, which leads to fever, abdominal pain, headaches and personality changes (Roop et al., 2004). Like *Listeria, Brucella* can survive and multiply in milk (Falenski et al., 2011), also following contamination after pasteurisation (Oliver et al., 2005). Due to the severe nature of many of the illnesses caused by pathogens borne in milk, it is important to test rigorously for their presence. While traditional methods can be laborious and time consuming, newer culture-independent methods have been investigated, with quantitative PCR being particularly rapid and sensitive (Quigley et al., 2011). However, these have yet to be implemented on a large scale by the dairy industry. While detection is important, the practices that prevent or limit the presence of pathogens are more crucial. Thus, it is important to implement a hygiene system that begins at the farm level and includes a focus on cow health and hygiene, equipment cleanliness, overall farm and personnel sanitation, correct storage and subsequent processing of milk.

Mycotoxins, *i.e*. low-molecular-weight compounds produced as secondary metabolites by filamentous fungi, can lead to illness in humans with symptoms such as nausea, vomiting, diarrhea and headache (Creppy, 2002) and some mycotoxins have carcinogenic potential (Murphy et al., 2006) The most important genera of food mycotoxigenic fungi are *Aspergillus, Alternaria, Fusarium* and *Penicillium*. Examples of mycotoxins of greatest public health and agro-economic significance include aflatoxin, trichothecenes, zearalenone, fumonisins and ochratoxin. After their intake by cows, mycotoxins follow a typical pharmacokinetic cascade of uptake from the gastrointestinal tract to the blood, internal distribution, metabolism, storage, remobilization and excretion. The rumen has an important function in the metabolism of mycotoxins with some mycotoxins being rapidly metabolised to less toxic metabolites (e.g. ochratoxin); some are transformed into equally toxic or more toxic metabolites (e.g. zearalenone), while some are not transformed at all (e.g. fumonisins). Aflatoxin B_1 is transformed into aflatoxin M_1 in the liver of ruminants. While M_1 is less mutagenic and genotoxic than B_1, the cytotoxicity of M_1 and B_1 is similar. Notably, aflatoxin B_1 is the only mycotoxin with significant carry-over into milk. Between 1 - 6% is excreted in milk, as aflatoxin M_1. Sixty countries now have regulations with respect to the presence of aflatoxin M_1 in milk, with limits of 0.05 - 0.5 μg kg^{-1}, the EU has a legal limit of 50 ng L^{-1} (Driehuis, 2013). Testing is important given that one study has shown that a high percentage (83.2%) of raw milk samples in Portugal were positive for aflatoxins (Martins and Martins, 2000) and that the levels of aflatoxin B_1 may frequently exceed recommended limits (Nordkvist and Hoorfar, 2012). Finally, yeasts, and especially *Candida* species, can be opportunistic pathogens, causing infections in immunocompromised patients. *D. hansenii* and *Y. lipolytica* may also be emerging dairy pathogens. These microorganisms cause rare infections in immunocompromised patients, which are generally mild and either self-limiting or easily treated (Jacques and Casaregola, 2008).

8.2 Antibiotic residues and antibiotic resistant bacteria in milk

Antibiotics have been employed to treat bacterial diseases over the past 70 years. The greatest threat to the successful application of antibiotics has been

the development of resistance, particularly in pathogenic bacteria. Resistance can be intrinsic or acquired. Intrinsic resistance is a natural characteristic of a microorganism that allows it to grow in the presence of the corresponding antibiotic. Acquired resistance results either from spontaneous mutation in the bacterial genome or from the acquisition of genes encoding resistance through transduction, conjugation or transformation (Davies, 1997).

Although lactococci, enterococci and lactobacilli are intrinsically resistant to some antibiotics (Mathur and Singh, 2005), the strains of these that are found in foods are typically quite sensitive to clinically important antibiotics such as ampicillin, penicillin, gentamicin and vancomycin (Franz et al., 1999, Herreros et al., 2005, Mannu et al., 2003, Mathur and Singh, 2005, Čanžek Majhenič et al., 2005). Furthermore, *Leuconostoc* strains are generally sensitive to antibiotics (Swenson et al., 1990). However, it is still important to assess the frequency with which antibiotic-resistant isolates occur in milk. A recent study determined that psychrotrophs, including *P. fluorescens, P. aeruginosa, Stenotrophomonas maltophila, Burkholderia* species, as well as a number of unidentified psychrotrophs, isolated from milk harbour resistance to several β-lactam and non-β-lactam antibiotics. This trait appears to increase in occurrence through the cold chain transportation of raw milk (Munsch-Alatossava and Alatossava, 2007). Bacteria of the genus *Acinetobacter* isolated from raw milk have also exhibited antibiotic resistance (Gurung et al., 2013). These bacteria are widespread in nature, including soil and water and are opportunistic pathogens in humans; multi-drug resistant strains are a serious concern (Dijkshoorn, 2013). Other antibiotic-resistant raw milk isolates include *L. lactis* displaying resistance to tetracycline, clindamycin and erythromycin and *L. garvieae* exhibiting resistance to tetracycline, streptomycin and quinupristin-dalfopristin (Walther et al., 2008).

The use of antibiotics to treat animals that are in the food chain can obviously compound this issue by selecting for the development of antibiotic resistance among food microorganisms (in particular in cow's milk) and by exposing consumers to antibiotic residues in milk and other dairy foods (Doyle et al., 2013). The use of antibiotics to treat mastitis during lactation is common, as between 2% and 55% of cow's encounter a mastitis infection during this period (Kelton et al., 1998). Notably, bacterial strains associated

with bovine mastitis, including many *S. aureus* isolates, have demonstrated resistance to antibiotics such as penicillins, oxytetracycline, streptomycin and/or gentamicin (Thaker et al., 2013). These problems can be limited through the withholding of milk from sale in situations where a cow has mastitis, is being treated with antibiotics or during a compulsory withdrawal period after antibiotic treatment. Safeguards, such as those introduced by Codex Alimentarius 2009 and European Union Council Regulation 37/2010/EC, require the monitoring of milk and provide limits with respect to the concentration of antibiotic residues that are tolerated in milk for commercial use.

8.3 Health-promoting microbes

Some raw milk isolates can have health promoting abilities; for millennia it has been suggested that fermented milk can cure some disorders of the digestive system and biblical scriptures highlight the use of milk to treat body ailments (Lourens-Hattingh and Viljoen, 2001). In 1907, the Russian scientist Elie Metchnikoff pointed out the benefits of consuming a diet of fermented milk (Rasić and Kurmann, 1983). Health-promoting bacteria isolated from these beverages and other sources are commonly referred to as “probiotics”, *i.e.* “live bacteria which when administered in adequate amounts confer a health benefit on the host” (FAO/WHO, 2002). The selection of such bacteria for commercial probiotic application relies on criteria relating to safety, technological and digestive stress survival, intestinal cell adhesion and human origin. The latter two criteria are controversial and it is now recognised that adhering to these criteria should not be mandatory, though may be desirable in certain instances. Notably, many raw milk isolates have desirable probiotic traits. These include the ability to survive bile juice, to tolerate gastric acid conditions and to adhere to intestinal cells (Jamaly et al., 2011). Probiotic lactobacilli typically inhibit pathogenic organisms, reduce lactose intolerance, increase the immune response and often are gastrointestinal isolates (Maragkoudakis et al., 2006, Kopp-Hoolihan, 2001). However, there are a number of dairy lactobacilli isolates which also have demonstrated efficiency as probiotic strains (Maragkoudakis et al., 2006). Other milk and dairy isolates that exhibit probiotic properties include strains of *L. lactis* as well as a variety

of *Pediococcus, Leuconotoc, Enterococcus* and *Streptococcus* isolates (Forghani et al., 2012, Kim et al., 2006, Espeche et al., 2012, Floros et al., 2012, Premalatha and Dhasarathan, 2011). Strains of *Propionibacterium freudenreichii*, and to a lesser extent *P. acidipropionici*, have begun to attract attention as potential probiotics as a consequence of studies revealing an ability, either alone or in combination with other probiotics, to reduce pathogen adhesion to mucus (Collado et al., 2008), increase bifidobacteria counts in the gut, aid in restoring a healthy gut microbiota, improve bowel movement, alleviate inflammatory disorders and reduce allergy development in infants (Cousin et al., 2012, Jan et al., 2002). Finally, and from a fungal perspective, the dairy yeast *Pichia fermentans* has demonstrated some probiotic potential and it has been suggested that, together with strains of *P. kudriavzevii* and *Yarrowia lipolytica*, *P. fermentans* could serve as probiotics that assimilate cholesterol (Chen et al., 2010). Regardless of the specific microbe in question, dairy products are an excellent vehicle for probiotics, regardless of their source, due to their buffering capacity and fat content, which can help protect the bacteria during gastric transit.

There has been quite a degree of focus on the use of dairy microbes to control hypertension. The rennin–angiotensin–aldosterone system is a key factor in the maintenance of arterial blood pressure. One of the main components of this system is angiotensin-converting enzyme (ACE). As ACE plays an important role in the regulation of arterial blood pressure, inhibition of this enzyme can generate an antihypertensive effect. ACE-inhibitory drugs are commonly used to control arterial blood pressure. Raw cow's milk can be a source of anti-hypertensive activity (Meisel, 2005). LAB that release bioactive peptides with this activity include strains of *Ent. faecalis*, *L. lactis ssp. cremoris, L. helveticus, L. fermentum, L. rhamnosus, L. paracasei* and *L. acidophilus* (Muguerza et al., 2006). The antihypertensive properties of these microbes are being investigated and exploited by industry with a view to producing health-promoting drinks. Indeed, *L. helveticus* is currently used in the production of fermented drinks such as Evolus (Valio Ltd., Valio, Finland) and Calpis (Calpis Food Industry Co. Ltd., Tokyo, Japan), which have properties associated with a reduction of blood pressure through the inhibition

of ACE as a consequence of the production of bioactive tripeptides (Slattery et al., 2010).

Raw milk and the raw milk microbiota have also been the focus of attention with respect to alleviating allergy. Allergy to cow's milk affects 2.5% of children below 3 years of age due to the presence of caseins and β-lactoglobulins (Cocco et al., 2003, Gaudin et al., 2008). The bacterial fermentation of milk proteins, particularly by highly proteolytic *Lactobacillus* populations, results in a reduction in the allergenic properties of cow's milk (El-Ghaish et al., 2011). Others have suggested a link between farm living, including the consumption of raw milk and raw milk microbes, and protection against the development of asthma and atopy later in life (Debarry et al., 2007, Ege et al., 2012). If confirmed, further investigations will be required to determine if overall microbial load or specific components of the microbial population are responsible.

9. Conclusion

The microbial community within raw milk is complex. The dominant, and subdominant, microorganisms present in raw milk can have a variety of influences on the flavour, taste and texture of raw milk-derived products (Figure 1). A number of these microorganisms also have the potential to contribute to health through the production of antimicrobials or possessing other probiotic-associated traits. Through modern genomics-based analysis it has been established that many of these microbes have become adapted to milk niches from various sources, including plant and gut environments, through genomic evolution and gene gain and/or loss. Despite the beneficial impact of many milk-associated microbes from a flavour, technological or health-related perspective, it is clear that there can be significant risks associated with the consumption of raw milk and raw milk-derived products or, more specifically, of the pathogens that can be found therein. While many of these microorganisms gain entry to the milk from equipment and/or personnel, zoonotic pathogens can also be introduced into milk from unhealthy animals. As a consequence of this risk, pasteurisation or other treatments are employed to remove disease-causing microorganisms. In the food industry, the negative impact of removing LAB and other bacteria on subsequent food fermentations has been addressed for some time through their reintroduction in the form of starter and adjunct cultures. Similarly, once established definitively, it may be possible to restore the benefits associated with the consumption of raw milk, and specific microorganisms therein, through the reintroduction of these microorganisms after processing. Thus, the microbial composition of raw milk is likely to continue to be the focus of much attention into the future.

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Figure 1: The potential sources of the microorganisms that are present in raw milk and the role/significance that some of these have when present in milk.

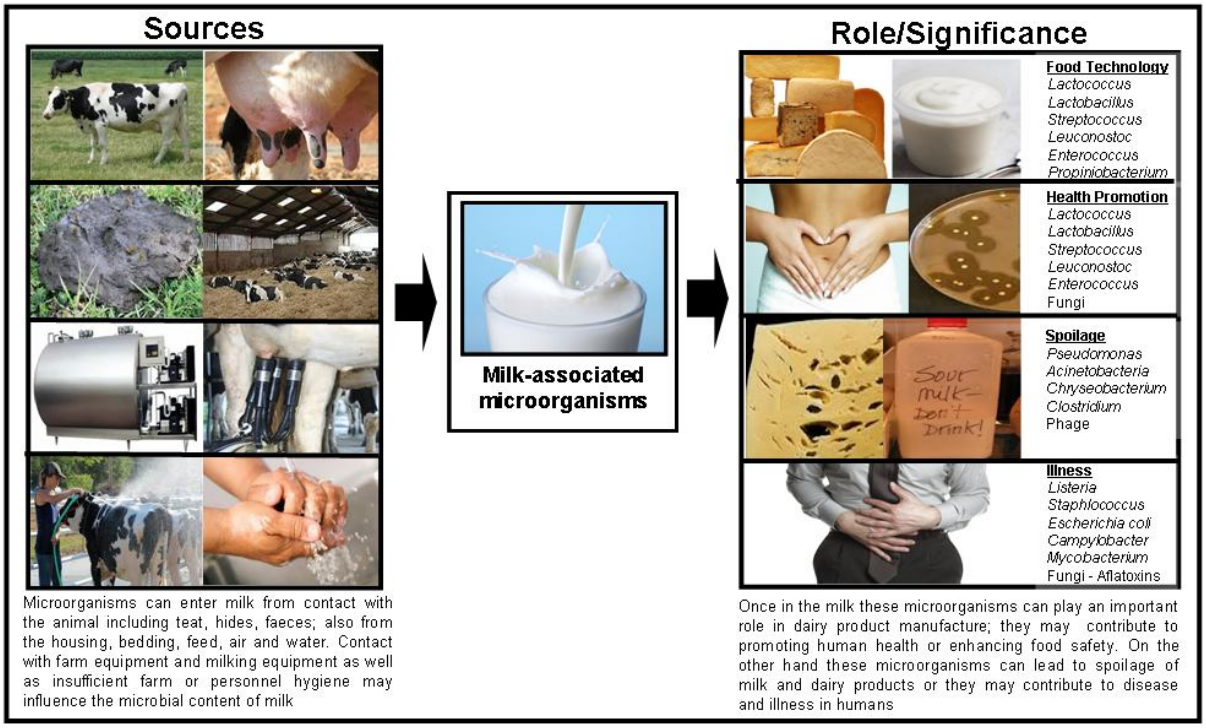
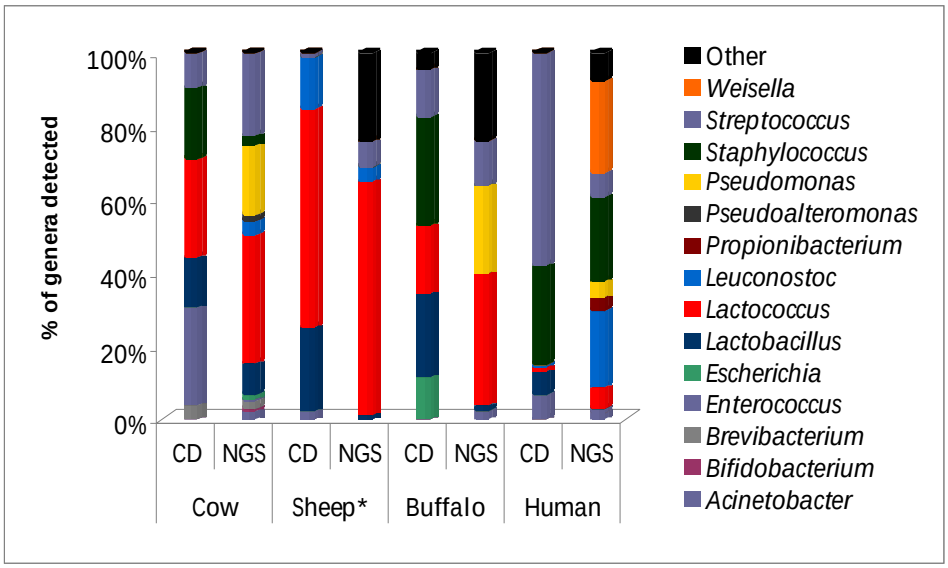


Figure 2: Relative abundance** of the most common bacteria in raw milk samples as detected by culture-dependent (CD) or next-generation DNA sequencing (NGS)-based technologies. CD results are represented as a percentage of total isolates. NGS results are represented as a percentage of total reads. The minor bacterial populations and species detected can be viewed in the tables corresponding to each milk type found throughout the manuscript and, in particular, Tables 1-4.



* Sheep's milk information was extrapolated from data relating to naturally ripened cheeses manufactured using raw sheep's milk.

**This graph provides merely an overview derived from a number of separate studies (Alegria et al., 2012, Cabrera-Rubio et al., 2012, Delbes et al., 2007, Devirgiliis et al., 2008, Ercolini et al., 2012, Heikkila and Saris, 2003, Hunt et al., 2011, Maniruzzaman et al., 2010, Masoud et al., 2011, Quigley et al., 2013) using different methods and from various locations. To more accurately assess the outputs generated by these respective approaches one should assess identical samples using both approaches.

Table 3: Bacterial populations detected in raw cow's milk using culture-dependent, -independent and next-generation DNA sequencing methods.

Cow's Milk	Culture-Dependent▲	Culture-Independent ▵	Next-Generation Sequencing+
(Mallet et al., 2012, Masoud et al., 2012, Quigley et al., 2013, Raats et al., 2011, Vacheyrou et al., 2011, Verdier-Metz et al., 2009)	*Acinetobacter species/johnsonii/junii/haemolyticus/lwoffii*	*Acinetobacter* species/*johnsonii/baumanii/junii*	*Acinetobacter* species
	Aerococcus species/*viridans*	*Aerococcus*	*Aerococcus* species
	Bacillus species/*cereus*	*Bacillus thuringiensis*	*Bacillus subtilis*
	Brevibacterium helvolum/linens	*Brevibacterium* species/*samyangensis*	*Brevibacterium linens*
	Chryseobacterium species	*Chryseobacterium* species/*joostei/bifermentans/freundii*	*Chryseobacterium piscium*
	Corynebacterium ammoniagenes/freneyi/glutamicum/variabilis/casei	*Corynebacterium freneyi/casei/variabile/macginleyi*	*Corynebacterium casei*
	Enterococcus species/faecalis/gallinarium/saccharominimus	*Enterococcus aquimarinus*	*Enterococcus faecalis*
	Escherichia coli	*Escherchia coli*	*Escherichia coli*
	Lactobacillus casei/curvatus/mindensis/animalis/coryneformis/curvatus/delbrueckii/johnsonii/paracasei/paraplantarum/plantarum/rhamnosus/amylovorus	*Lactobacillus plantarum/pentosus/delbrueckii/acidophilus/fermentum*	*Lactobacillus casei/ helveticus/plantarum/sakei/rhamnosus*
	Lactococcus lactis/garviae	*Lactococcus lactis/garvieae*	*Lactococcus lactis*
	Leuconostoc mesenteroides	*Leuconostoc carnosum/pseudomesenteroides*	*Leuconostoc*
	Staphylococcus species/*capitis/cohnii/saprophuticus/equorum/xylosus/aureus/haemolyticus/hominis/epidermis*	*Staphylococcus aureus/epidermidis/fleuretii/sciuri*	*Staphylococcus saprophyticus/succinus*
	Streptococcus species/*uberis/parauberis*	*Streptococcus species/uberis/dysgalaciae/parauberis/thermophilus*	*Streptococcus thermophilus*

Table 1 continued:

Cow's Milk	Culture-Dependent▲	Culture-Independent ∆	Next-Generation Sequencing┼
	Pseudomonas species/alcalophila/stutzeri/synxantha/fluroscens/putida	*Pseudomonas species/fragi/psychrophila/brenneri/synxantha/putida/pertucinogena*	*Pseudomonas species/gessardii*
	Microbacterium liquefaciens/oxidans/lacticum	***Microbacterium species/xinjiangensis***	*Macrococcus equipercicus*
	Rhodococcus erythropolis	***Rothia***	***Rothia mucilaginosa***
	Serratia liquefaciens/odorifera	***Stenotrophomonas species/koreensis***	***Stenotrophomonas maltophilia***
	Enterobacter* species/*gergoviae	*Empedobacter brevis*	***Enterobacter cloacae***
	Klebsiella ozanae/oxytoca	***Klebsiella oxytoca***	*Kurthia gibsonii*
	Kocuria* species/*carniphila/kristinae/rhizophila	***Kocuria species/pneumoniae***	*Leptotrichia hofstadii*
	Frigoribacterium species	***Facklamia***	***Facklamia tabacinasalis***
	***Paracoccus* species**	*Nocardioides dubius*	***Paracoccus carotinifaciens***
	Micrococcus species	*Ornithinicoccus species*	*Marinomonas*
	Ochrobactrum anthropi/tritici	*Pandoraea species/norimbergensis*	*Meiothermus species*
	Panteoa species/*agllomerans*	*Phyllobacterium myrsinacerum*	*Methylobacterium extorquens*
	Propionibacterium freudenreichii/jensenii	***Propionibacterium***	*Pediococcus pentosaceus*
	Providencia stuartii	*Proteobacteria*	*Prevotella*
	Psychrobacter* species/*maritimus	*Ralstonia species/picketti*	***Psychrobacter***
	Pseudoclavibacter species/*helvolus*	*Sphingomonas melonis*	*Pseudoalteromonas agarivorans*
	Rahnella aquatilis	*Thauera species*	*Ruminococcus flavefaciens*
	Renibacterium salmoninarum	*Trichococcus*	*Weisella hellenica*
	Sphingomonas species	*Yania halotolerans*	*Actinomyces radicidentis*
	Achromobacter delictulus	*Acidobacteria*	*Alistipes finegoldii*
	Aeromonas hydrophila	*Adhaeribacter aquaticus*	***Aeromonas species/hydrophila/popoffii***
	Arthrobacter species/arilaitensis/psychrolactophilus	***Arthrobacter species/arilaitensis/psychrolactophilus***	*Anaerococcus actavius*
	Brachybacterium species/nesterenkovii	*Bacteroidetes*	*Bacteroides*
	Deinococcus species	*Bosea thiooxidans*	*Bifidobacterium species/pseudolongum*
	Dermacoccus species	*Bradyrhizobium species*	*Carnobacterium species*
	Hafnia alvei	*Caryophanon latum*	*Empedobacter brevis*

Table 1 continued:

Cow's Milk	Culture-Dependent▲	Culture-Independent Δ	Next-Generation Sequencing┼
	Clavibacter michiganensis	*Delftia*	*Catenibacterium*
	Comamonas testosteroni	*Clostridium perfringen/lituseburense*	*Caulobacter crescentus*
	Enhydrobacter aerosaccus	*Janibacter anophelis*	*Faecalibacterium*
	Halomonas species	*Janthinobacterium lividum*	*Jeotgalicoccus psychrotrophilus*
	Leucobacter species	*Paenibacillus apiarius*	**Unassigned**
		Unassigned	

▲Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.

Δ Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.

┼ NEXT-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.

The bacterial names emphasised in blue highlight the less prevalent but frequently isolated bacterial populations detected.

The bacterial names in black highlight the occasional bacteria which are detected.

Where there are bacterial names in **bold** these are detected by two of the three methods.

Where the bacterial name is underlined these were detected by only one of the methods.

All other bacteria were detectable by all of the methods.

Table 4: Bacterial populations detected in raw goat's milk using culture-dependent, -independent and next-generation DNA sequencing methods.

Goat's Milk	Culture-Dependent▲	Culture-Independent ▲	Next-Generation Sequencing+
(Callon et al., 2007; Goetsch et al., 2011;Alonso-Calleja et al., 2002)	*Acinetobacter baumanii* *Arthrobacter* species *Bacillus thuringiensis-cereus* *Brachybacterium paraconglomeratum* *Brevibacterium stationis* *Chryseobacterium indologenes* *Citrobacter freundii* *Corynebacterium variable* *Delftia acidovorans* *Enterococcus faecalis/saccharominimus* *Exiguobacterium* *Jeogalicoccus psychrophiles* *Kocuria rhizophila-Kristinae carniphila* *Lactobacillus casei* *Lactococcus lactis/garvieae* *Leuconostoc mesenteroides* *Microbacterium oxydans* *Micrococcus* species/*caseolyticus* *Pantoea agglomerans* *Pseudomonas* species/*putida/aeruginosa/ fulgida* *Salinicoccus* species *Staphylococcus epidermidis/simulans/caprae/equorum* *Stenotrophomonas maltophilia* *Streptococcus mitis*	*Acinetobacter baumanii* *Arthrobacter* species *Bacillus thuringiensis-cereus* *Brachybacterium paraconglomeratum* *Brevibacterium stationis* *Chryseobacterium indologenes* *Citrobacter freundii* *Corynebacterium variable* *Delftia acidovorans* *Enterococcus faecalis/saccharominimus* *Exiguobacterium* *Jeogalicoccus psychrophiles* *Kocuria rhizophila-Kristinae carniphila* *Lactobacillus casei* *Lactococcus lactis/garvieae* *Leuconostoc mesenteroides* *Microbacterium oxydans* *Micrococcus* species/*caseolyticus* *Pantoea agglomerans* *Pseudomonas putida/aeruginosa* *Salinicoccus* species *Staphylococcus epidermidis/caprae/simulans/equorum* *Stenotrophomonas maltophilia* *Streptococcus mitis* *Dietza maris* *Enterobacter* species/*absuria* *Hahella chejuinsis* *Klebsiella milletis-oxytoca* *Ornithinicoccus* species *Rothia* species	N/A

▲Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.

Δ Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.

┼ NEXT-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.

N/A – Not Applicable; at the time of writing this article next-generation DNA sequencing technology had not been applied to monitor the microbial content of raw goat milk or any source which may represent the microbial content of raw goat milk.

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.

The bacterial names emphasised in blue highlight the less prevalent but frequently isolated bacterial populations detected.

The bacterial names in black highlight the occasional bacteria which are detected.

Where there are bacterial names in **bold** these are detected by two of the three methods.

Where the bacterial name is underlined these were detected by only one of the methods.

All other bacteria were detectable by all of the methods.

Table 5: Bacterial populations detected in raw milk using culture-dependent, -independent and next-generation DNA sequencing methods.

Milk Source	Culture-Dependent▲	Culture-Independent ∆	Next-Generation Sequencing┼
Sheep's Milk*			
(Alegria et al., 2012)	*Lactococcus lactis* *Lactobacillus casei/plantarum/parabuchneri/ brevis* *Leuconostoc citreum/lactis/mesenteroides/ pseudomesenteroides* *Streptococcus thermophilus/agalactiae* *Enterococcus faecalis/durans/italicus/kobei* *Bacillus simplex* *Pediococcus* *Corynebacterium* species *Staphylococcus* species *Salmonella* species *Escherichia coli* *Enterobacter kobei*	*Lactoccus lactis/garvieae/raffinolactis* *Lactobacillus plantarum/paraplantarum/ helveticus/crispatus* *Leuconostoc citreum/mesenteroides-pseudomesenteroides/lactis* *Streptococcus vestibularis/salivarius/uberis-parauberis/thermophilus* *Enterococcus* ***Tetragenococcus halophilus*** *Bifidobacterium* *Chromohalobacter*	*Lactococcus* *Lactobacillus* *Leuconostoc* *Streptococcus* *Enterococcus* ***Tetragenococcus*** *Actinobacteria* *Chryseobacterium* *Enhydrobacter* *Flavobacteria* *Kocuria* *Sanguibacter* *Staphylococcus* Unassigned
Buffalo Milk			
Devirgiliis et al., 2008, Ercolini et al., 2001, Maniruzzaman et al., 2010; Ercolini et al., 2012)	*Lactobacillus species/plantarum/paracasei/ fermentum/delbrueckii* *Lactococcus* species *Staphylococcus* species Streptococcus *Bacillus* species *Escherichi coli* Unassigned	*Lactobacillus* species/*delbrueckii* *Lactococcus lactis* *Leuconostoc lactis* *Streptococcus thermophilus* *Enterococcus* species/*faecalis*	*Lactobacillus* species/*kefiranofaciens* *Lactococcus* species/*lactis* *Pseudomonas* species/*fragi* *Streptococcus thermophilus/macedonicus* *Acinetobacter* species/*johnsonii* *Carnobacteria* species *Clostidium species/hiranonis*

Table 3 continued:

Milk Source	Culture-Dependent▲	Culture-Independent Δ	Next-Generation Sequencing┼
			Corynebacteria species
			Enterobacteria species
			Unassigned

▲Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.

Δ Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.

┼ NEXT-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.

* Sheep's milk information is from naturally ripened cheeses manufactured using raw sheep's milk indicating that the microbial population is potentially from the raw sheep's milk microflora.

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.

The bacterial names emphasised in blue highlight the less prevalent but frequently isolated bacterial populations detected.

The bacterial names in black highlight the occasional bacteria which are detected.

Where there are bacterial names in **bold** these are detected by two of the three methods.

Where the bacterial name is underlined these were detected by only one of the methods.

All other bacteria were detectable by all of the methods.

Table 6: Bacterial populations detected in raw human milk using culture-dependent, -independent and next-generation DNA sequencing methods.

Human Milk	Culture-Dependent▲	Culture-Independent ▲	Next-Generation Sequencing┼
(Cabrera-Rubio et al., 2012;Hunt et al., 2011; Fernandez et al., 2013)	*Bifidobacterium* species/*adolescentis/ bifidum/breve/longum*	*Bifidobacterium* species/*longum/ adolescentis/animalis/bifidum/breve/ catenolatum*	*Bifidobacterium*
	Corynebacterium species	*Corynebacterium* species	*Corynebacterium*
	Enterococcus species/ *faecium/faecalis/durans/hirae/mundtii*	*Enterococcus faecalis/faecium*	*Enterococcus*
	Lactobacillus species/*acidophilus/ fermentum/plantarum/gasseri/crispatus/rha mnosus/salivarius/reuteri/casei/gastricus/va ginalis/animalis/brevis/helveticus/oris*	*Lactobacillus* species/*fermentum/ gasseri/rhamnosus*	*Lactobacillus*
	Lactococcus species/*lactis*	*Lactococcus* species/*lactis*	*Lactococcus*
	Leuconostoc species/*mesenteroides*	*Leuconostoc* species/*citreum/fallax*	*Leuconostoc*
	Streptococcus species/*mitis/salivarius/ oris/parasanguis/lactarius/australis/ gallolyticus/vestibularis*	*Streptococcus* species/*mitis /parasanguis/salivarius*	*Streptococcus*
	Staphylococcus species/ *epidermidis/aureus/capitis/hominis*	*Staphylococcus* species/*epidermidis/hominis*	*Staphylococcus*
	Rothia* species/*mucilaginosa	*Clostridium* species	***Rothia***
	Pediococcus species/*pentosaceus*	***Weisella* species/*cibaris/confusa***	***Weisella***
	Peptostreptococcus species	***Propionibacterium* species/*acnes***	***Propionibacterium***
	Kocuria species/*rhizophilia*	***Acinetobacter***	***Acinetobacter***
		Pseudomonas	***Pseudomonas***
			Sphingomonas
			Ralstonia
			Serratia
			Stenotrophomonas
			Veillonella
			Actinomyces

Table 4 continued:

Human Milk	Culture-Dependent▲	Culture-Independent Δ	Next-Generation Sequencing┼
			Bradyrhizobium *Carnobacterium* *Citrobacter* *Gemella* *Granulicatella* *Lysinibacillus* *Prevotella* Unassigned

▲Culture-dependent methods are based on isolation of bacteria using agar-based methods followed by identification using phenotypic or genotypic methods.

Δ Culture-independent methods are based on direct extraction of bacterial DNA from the milk followed by identification using various techniques including DGGE/SSCP/clone libraries etc.

┼ NEXT-generation sequencing methods are based on direct extraction of bacterial DNA from the milk sample followed by identification using pyrosequencing.

The bacterial names emphasised in red highlight the most prevalent bacterial populations detected.

The bacterial names emphasised in blue highlight the less prevalent but frequently isolated bacterial populations detected.

The bacterial names in black highlight the occasional bacteria which are detected.

Where there are bacterial names in **bold** these are detected by two of the three methods.

Where the bacterial name is underlined these were detected by only one of the methods.

All other bacteria were detectable by all of the methods.

Supplementary Data

Table S1: Representative nutritional content of four major milk types consumed throughout the world.

Nutritional Content of Milks				
	Cow	**Goat**	**Sheep**	**Human**
Crude Proteins g/100g				
Total Casein*	2.63	2.47	3.98	0.4
Alpha-Casein	44.8	39.82	57.4	11.7
Beta-casein	35.7	51.16	5.06	64.7
kappa-casein	12.6	9.02	13.3	23.5
Total Whey Proteins**	0.57	0.52	0.92	0.76
Beta-lactoglobulin	20	22.9	17	Absent
Alpha-lactalbumin	53.6	37	42	42.4
Immunoglobulin	11.7	4.9	4.5	18.1
Serum albumin	0.04	0.11	0.06	7.5
Lactoferrin	8.3	6.2	6.8	30.2
Lysozyme	Trace	Trace	Trace	1.6
Carbohydrates g/100g				
Lactose	4.8	4.1	3.7	6
Oligosaccharides	Trace	Trace	Trace	0.5-0.8
Total Fats g/100g				
Saturated	2.08	2.67	4.6	1.8
Monounsaturated	0.96	1.11	1.71	1.6
Polyunsaturated	0.12	0.15	0.32	0.5
Cholesterol (mg/100g)	10	11	27	9
Vitamins/100g				
Retional (mg)	0.04	0.04	0.08	0.06
Beta Carotene (mg)	0.02	absent	absent	0.02
Other B Viatmins (mg)	0.04-0.35	0.05-0.31	0.08-0.71	0.01-0.18
Folic Acid (µg)	5.3	1	5	5.2
Biotin (µg)	0.7	2	absent	0.7
Vitamin C (mg)	1	1.3	5	4
Minerals /100g				
Calcium (mg)	120	126	195-200	32
Iron (µg)	22	30	40-68	36
Magnesium (mg)	11	13	18-21	4
Phosphorus (mg)	92	97	124-158	15
Potassium (mg)	150	190	136-140	55
Sodium (mg)	45	38	44-58	20
Zinc (µg)	380	340	520-747	300
Copper (µg)	22	30	40-68	36
Manganese (µg)	6	8	5.3-9.0	3
Selenium (µg)	3	2	3.1	2
Iodine (µg)	7	8	10.4	8
Total Amino Acids g/L ***				
Arginine	34	29	34	36
Serine	56	49	52	61
Aspartic Acid	70	75	75	86
Threonine	42	49	41	44
Glycine	18	18	18	22
Alanine	32	34	40	40
Tyrosine	47	38	47	46
Proline	100	106	102	95

Table S1 continued:

Nutritional Content of Milks				
	Cow	**Goat**	**Sheep**	**Human**
Methionine	26.3	25.5	28.7	16.1
Valine	52	61	57	51
Phenylalanine	50	47	48	37
Isoleucine	47	48	49	53
Leucine	99	96	90	104
Histidine	24	26	26	23
Lysine	86	80	83	71
Glutamate	208	209	203	190
Cystine	8.9	8.6	7.5	20.2

* Individual casein values are represented as a % of total casein content.

** Individual whey protein components are represented as a % of total whey protein content.

*** Individual Amino Acid values are calculated in mg/g as a concentration of total amino acids (except Tryptophan).

This table was compiled from the studies of (Davis et al., 1994, Raynal-Ljutovac et al., 2008).

Chapter I

A comparison of methods used to extract bacterial DNA from raw milk and raw milk cheese

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Abstract

In this study we compare seven different methods which have been designed or modified to extract total DNA from raw milk and raw milk cheese with a view to its subsequent use for the PCR of microbial DNA. Seven extraction methods, five solid-phase extraction and two liquid-liquid phase extraction methods were employed to extract total DNA from these foods and their relative success with respect to the yield and purity of the DNA isolated, and its quality as a template for downstream PCR reaction, was compared. Although all of the methods were successful with respect to the extraction of total DNA naturally present in cheese, they varied in their relative ability to extract total DNA from milk. However, when milk was spiked with a representative Gram-positive (*Listeria monocytogenes* EGDe) or Gram-negative (*Salmonella enterica* serovar Typhimurium LT2) bacterium, it was established that all methods successfully extracted DNA which was suitable for subsequent detection by PCR. Of the seven approaches, the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) was found to most consistently extract highly concentrated and pure DNA with a view to its subsequent use for PCR-based amplification and also facilitated accurate detection by real-time quantitative PCR. Accurately assessing the microbial composition of milk and cheese is of great importance to the dairy industry. Increasingly, DNA-based technologies are being employed to provide an accurate assessment of this microbiota. However, these approaches are dependent on our ability to extract DNA of sufficient yield and purity. This study compares a number of different options and highlights the relative success of these approaches. We also highlight the success of one method to extract DNA from different microbial populations as well as DNA which is suitable for real-time PCR of microbes of interest, a challenge often encountered by the food industry.

1. Introduction

Until recently, our understanding of the composition of microbial ecosystems has been limited by a reliance on culture-based techniques (Hugenholtz et al., 1998). However, in the last decade DNA-based methods have been developed which have provided an alternate, culture-independent, means of analysing such communities (Nocker et al., 2007). It has been established that DNA-PCR based methods are highly specific, reproducible and sensitive and are characterised by high discriminatory power, rapid processing time and low costs and thus have been employed to investigate the microbial composition of foods (Di Pinto et al., 2007). Unfortunately, food samples frequently contain PCR inhibitors such as fats, proteins and calcium that can compromise the amplification of DNA (Wilson, 1997). It has been reported that obtaining DNA extracts from dairy products which are non-degradable, inhibitor-free and suitable for PCR amplification is a common problem (Pirondini et al., 2010). For these reasons, extracting DNA of sufficient concentration and purity is of crucial importance. The methods used to extract and purify DNA from foods frequently consist of four key steps, i.e. mechanical homogenisation, treatment with buffers, detergents and/or enzymes, the application of mechanical lysis steps and the organic extraction of DNA (Jany and Barbier, 2008). With respect to mechanical homogenisation, there are a number of existing procedures which employ a stomacher, Pulsifier® (Fung et al., 1998), blender (Parayre et al., 2007) or similar such pieces of equipment. These, in the presence of salt based buffers such as tri-sodium citrate, NaOH based media or detergent based buffers, macerate the food sample thereby releasing microorganisms into suspension (Callon et al., 2006). Once the released cells have been retrieved, they may be treated with buffers containing chaotrophic agents, for example guanidine thiocyanate and/or detergents such as SDS, which disorder the structure of DNA, helping to burst-open the cells and release DNA (Duthoit et al., 2003). Such buffers also contribute to a reduction in the concentration of inhibitory substances. Enzymes, such as proteinase K, lysozyme and mutanolysin may be used as these degrade the cell wall of more resilient microorganisms including Gram-positive species. Mechanical cell lysis, which breaks open the

bacterial cell wall by vibrating bacteria with microbeads at high speeds, has also been found to improve detection limits (Odumeru et al., 2001). DNA can then be extracted using organic solvents, such as phenol-chloroform, which aid in removing proteins and other cell remnants, before DNA purification and concentration, generally using an ethanol precipitation step (Duthoit et al., 2003, Giannino et al., 2009). However, more recently, commercial kits (Abriouel et al., 2006, Pirondini et al., 2010) relying on DNA-binding matrices or magnetic solid-phase supports have circumvented the need for dangerous chemicals such as phenol. The former generally use a column-based system which works on the basis of affinity chromatography, i.e. the DNA adsorbs to the membrane (e.g. silica-based), and all impurities are washed through with the DNA then being eluted from the membrane using a low-salt buffer (e.g. TE (Tris/EDTA) buffer). Magnetic-based approaches rely on the reversible binding of DNA, non-specifically to magnetic microparticles which have a DNA-binding functional group attached (Abriouel et al., 2008, Di Pinto et al., 2007).

Here we evaluate seven different methods which have been designed or adapted to facilitate the extraction of DNA from raw milk and raw milk cheese. This involves a comparison of the quality and yield of DNA isolated and an assessment of the success with which PCR amplicons are generated.

2. Materials and Methods

2.1 Food sources

Fresh milk samples were collected, in triplicate, from a milking parlour under aseptic conditions and immediately placed in isothermic conditions and transferred to the laboratory for DNA extraction. A commercial, soft, raw milk cheese manufactured from cow's milk with starter cultures was sampled, in triplicate, under aseptic conditions.

2.2 DNA extraction methods

Seven DNA extraction methods were evaluated to compare their relative efficiency with respect to the extraction of DNA from milk and cheese samples. The characteristics of the DNA extraction methods are summarized in Table 1. Methods 1-3 relied on the QIAamp® DNA stool mini kit (Qiagen Ltd., Crawley, West Sussex, UK), Chemagic Food Basic kit (Chemagen Biopolymer-Technologie AG, Baesweiler, Germany) and Wizard® Magnetic DNA purification system for Food (Promega Corporation, Madison, Wisconsin, USA) to extract DNA from 200 mg of cheese or a pellet from 1 ml milk. Extractions were carried out according to the manufacturers' instructions including a recommended modification to the QIAamp® protocol designed to enhance its ability to extract DNA from food matrices. Methods 4-5 employed the Milk Bacterial DNA Isolation kit (recommended by manufacturer's for extracting DNA from milk) (Norgen Biotek Corporation, Ontario, Canada), and the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc, Carlsbad, California, USA), to extract DNA, again from a pellet obtained from 1 ml of milk or 1 ml of cheese homogenate (prepared by stomaching 1 g cheese with 9 ml tri-sodium citrate), according to manufacturers' instructions. Method 6 was designated the 'Lytic' method and represents a combination of methods used by O'Mahony and Hill (2004), Parayre et al (2007) and Dolci et al (2008). Here DNA was isolated by resuspending the pellet (obtained from 1 ml milk or 1 ml of homogenised cheese) in 500µl of breaking buffer for enzymatic lysis (20mM Tris HCl (pH8), 2 mM EDTA, 2% Triton X100, 50 µg ml^{-1} lysozyme, 100U mutanolysin) and incubated at 37°C for 1 h. Protein digestion was then performed by adding 250 µg ml^{-1} proteinase K and

incubating at 55°C for 1 h. The suspension was transferred to a 2 ml tube containing 0.3 g zirconium beads, the tube was shaken for 90 sec in a bead beater, twice, and centrifuged at 12,000 *g* x 10 min. The supernatant was transferred to a fresh tube and combined with an equal volume of phenol:chloroform:isoamylalcohol (25:24:1) mixed gently and centrifuged at 12,000 *g* x 2 min. The top aqueous phase was transferred to a clean tube and one tenth the volume 3 M sodium acetate and 2 volumes of 100% ice-cold ethanol was added. The suspension was mixed gently and stored at -20°C overnight. The sample was centrifuged at 14,000 *g* x 10 min, the supernatant removed and the pellet was washed with 70% ice-cold ethanol followed by centrifugation at 12,000 *g* x 5 min and the pellet dried. The pellet was re-suspended in 100 µl TE buffer. Finally, a 'guanidine thiocyanate'-based method, as described by Duthoit et al., was employed (Duthoit et al., 2003).

2.3 DNA Quantification measurement

The quantity of DNA extracted by the different methods was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen Corporation, Carlsbad, California, USA) used in accordance with the manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA). The ND3300 excites in the presence of dsDNA bound with Picogreen® at 470nm and monitors emission at 525nm. DNA purity was assessed on the basis of absorbance at 260-280nm using the NanoDrop 1000 (Thermo Fisher Scientific Inc). An A260/280 ratio of 1.8-2.0 is indicative of high purity (Pirondini et al., 2010).

2.4 PCR amplification of the microbial community 16S rRNA gene

The DNA extracts were used as a template for PCR amplification of 16S rRNA tags (V4 region; 239 nt long) using universal 16S primers predicted to bind to 94.6% of all 16S genes i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The PCR reaction contained 25 µl GoTaq Green Master Mix (Promega), 1 µl of each

primer (10 pmol), 5 µl DNA template and nuclease-free dsH_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (G-Storm Ltd, Surrey, UK). The amplification programme consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles; denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. The PCR product was purified using the High Pure PCR Cleanup Micro Kit (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) and quantified again using the Quant-It™ Picogreen® dsDNA reagent and the Nanodrop™ 3300. Quality and quantity was also assessed visually following agarose gel electrophoresis.

2.5 Purification and amplification of DNA from pathogen-spiked milk

Raw milk was artificially contaminated with *Listeria monocytogenes* EGDe (DPC 6554) and *Salmonella enterica* serovar Typhimurium LT2 (*S. typhimurium*) (DPC 6048) at a level of 10^7, 10^5, 10^3 and 10^1 cfu ml^{-1}. DNA was extracted from a 1 ml sample of contaminated milk using the seven DNA extraction methods described above. The DNA extracts were used as a template for PCR amplification using species-specific primers (Table 2). The PCR reaction contained 25 µl GoTaq Green Master Mix, 1 µl of each primer (10 pmol), 5 µl DNA template and H_2O to give a final reaction volume of 50µl. PCR amplification was performed using a G-Storm thermal cycler. The amplification programme consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles; denaturation at 94°C for 30 sec, annealing at 60°C for 1 min or 55°C for 30 sec for *L. monocytogenes* and *S. typhimurium*, respectively, and extension at 72°C for 45 sec. A final elongation step at 72°C for 2 min was also included. Success of the extraction protocol to extract Gram-positive and Gram-negative bacteria was determined by visual examination following gel electrophoresis.

The suitability of extracted DNA for subsequent amplification by real-time PCR (qPCR) was assessed using species-specific primers and a SYBR Green 1 Master Mix (Roche) on the LightCycler® 480 platform (Roche). All PCR reactions were performed in triplicate. Amplicons were generated using species-specific primers (Table 2), targeting the *hlyA* and *invA* gene for *L.*

monocytogenes and *S. typhimurium*, respectively. These amplicons were series diluted to generate a standard curve construct from 10^1 to 10^8 gene copies. Real-time PCR was subsequently carried out on DNA generated from the spiked milk samples. The PCR reaction contained 10 µl SYBR Green 1 Master mix, 1 µl 10 pmol forward and reverse species-specific primers, 3 µl of nuclease-free water and 5 µl of DNA template. The cycling conditions were as follows: *L. monocytogenes*, 95°C for 5 min, 40 cycles of 95°C for 20 sec, 60°C for 20 sec and 72°C for 20 sec; and *S. typhimurium* , 95°C for 5 min, 40 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 20 sec. At the end of the cycle the instrument showed the melting temperature (T_m) of the produced amplicons. The T_m of the amplicons were compared with the T_m of standard curve constructs. When the T_m corresponded with the T_m from the positive standards we considered the reaction successful.

3. Results

3.1 Comparison of the yield and purity of DNA extracted from raw milk and a raw milk cheese using a variety of extraction methods

Seven methods, five solid-phase extraction and two liquid-liquid phase extraction (Table 1), were tested to compare their relative efficiency with respect to extracting DNA, on the basis of yield and purity, from raw milk and a soft, raw cow's milk cheese. The yield and purity of genomic DNA varied with each method (and are summarised in Table 3). With respect to DNA yield from milk, the PowerFood™ Microbial DNA Isolation kit, Milk Bacterial DNA Isolation kit and 'Lytic' method were most successful. The 'guanidine thiocyanate' method provided the lowest yield (Table 3). The corresponding purity values revealed that while the 'guanidine thiocyanate' yield was low, the quality of this DNA was very high, i.e. A260/280 ratio of 1.92. The PowerFood™ Microbial DNA Isolation kit and the QIAamp® DNA stool mini kit both also provided very pure DNA. Although the Milk Bacterial Isolation kit provided very good yields, the purity of this DNA was less impressive and the purity of DNA extracted by the Chemagic Food Basic kit, Wizard® Magnetic DNA Isolation kit and the 'Lytic' method were somewhat lower.

When the same methods were employed to extract DNA from a raw milk cheese, the highest yields were provided by the 'Lytic' method and the PowerFood™ Microbial DNA Isolation kit, here the 'guanidine thiocyanate' method also provided the lowest DNA yield. It was established that purity of the DNA generated by the PowerFood™ Microbial DNA Isolation kit and 'guanidine thiocyanate' methods was highest but that generated using the Wizard® Magnetic DNA Isolation kit was very low. In addition, the DNA extracted using the Chemagic Food Basic kit provided an excessively high A260/280 ratio of 2.3 (Table 3), which is indicative of the inefficient removal of organic contaminants (Viltrop et al., 2010). As with low purity values, high A260/280 ratios indicate the likelihood of downstream difficulties when utilising the extracted DNA.

3.2 Amplification of DNA extracted from raw milk and raw milk cheese

To further investigate the relative success of the seven extraction methods, the extracted DNA was used as a template for the amplification of 16S rRNA genes using universal PCR primers. Following their purification, the concentration of the PCR products generated was determined, again using the Quant-It™ Picogreen® dsDNA reagent/Nanodrop™ 3300 Fluorospectrometer. The concentration of the PCR products generated using DNA template extracted from milk varied considerably (Table 3) and, for example, DNA generated using the 'guanidine thiocyanate' method was not successfully amplified. However, all seven methods extracted DNA from cheese that was efficiently amplified (Table 3).

3.3 Recovery of Gram-negative and Gram-positive bacteria as assessed by conventional PCR

While the approach described above highlights the relative abilities of the different approaches with respect to the extraction of total microbial DNA, of unknown origin, with a view to its subsequent amplification, many of the pathogenic microbes which are of greatest concern with respect to raw milk and raw milk cheese are from the Gram-negative Proteobacteria and the low G-C Gram-positive Firmicutes. Therefore it was deemed important to determine the relative ability of the various kits to extract DNA to facilitate the PCR based detection of representatives of these phyla in milk. To facilitate this *Listeria monocytogenes* EGDe (a Firmicutes) and, *Salmonella typhimurium* LT2 (a Proteobacteria) were respectively introduced into raw milk at a range of levels between 10^7 and 10^1 cfu ml^{-1}. DNA was extracted using the seven extraction methods and the success of species-specific PCR assays (designed to amplify from *hlyA* and *invA*, respectively) was assessed. Visual examination of gel electrophoresis images revealed that PCR band intensity was greatest when template DNA was extracted using the Milk Bacterial Isolation kit, the PowerFood™ Microbial DNA Isolation kit and the 'Lytic' method (Figure 1). The Qiagen DNA stool mini kit, Chemagic Food Basic kit and the Wizard® Magnetic DNA purification system led to the efficient extraction and amplification of *S. typhimurium* DNA at all concentrations but were less successful when milk was spiked with *L*.

monocytogenes at low concentrations. The guanidine thiocyanate method performed poorly regardless of the target pathogen.

3.4 Improvement of DNA yield and PCR efficiency

Of the seven extraction methods, the PowerFood™ Microbial DNA Isolation kit and the 'Lytic' methods provided the most impressive results. Of these the former has the advantage of being more rapid and does not require the use of harmful chemicals such as phenol and, thus, became the focus of further attention. More specifically, investigations were carried out to determine if additional 'troubleshooting' steps provided within the manufacturer's instructions can further increase DNA yield and quality. Briefly, the respective success of four supplemental steps was assessed. These involved an additional heat treatment of samples at (i) 65°C or (ii) 70°C for 10 min prior to step 5 of the extraction process as described by the manufacturers, (iii) a 65°C heat treatment followed by the exclusion of the two subsequent steps of the process or (iv) the further concentration of DNA at the end of the process through ethanol precipitation. The success of a fifth modification, whereby the enzymatic treatment employed by the 'Lytic' method was introduced prior to step 5, was also assessed. In all cases the inclusion of additional steps did not further enhance the yield of DNA from cheese (Table 4). Indeed, in the case of the additional ethanol precipitation step, the final yield was greatly reduced from 9062 ng g^{-1} to 4673 ng g^{-1}. Although ethanol precipitation also impacted negatively on the yield of DNA from milk (439 ng g^{-1}), each of the other steps brought about a major increase in DNA yields (Table 4). However, a heat treatment at 70°C for 10 minutes followed by 10 minutes of vortexing was most successful in that a yield increase from 896 ng ml^{-1} to 3471 ng ml^{-1} resulted. The purity of the DNA extracted with these supplementary steps was measured. Again the results showed the ability of the PowerFood™ Microbial DNA Isolation kit to extract high quality, pure DNA with A260/280 readings ranging between 1.72 and 2.0 from both milk and cheese extracts.

Subsequent 16S rRNA gene amplification determined the suitability of DNA from the additional steps for PCR amplification. After purification of the resultant amplicons, its concentration was determined (Table 4). The concentration of the PCR products generated, using DNA template extracted

from milk, was considerable except in cases where ethanol precipitation was employed. More specifically, it was revealed that all other treatments led to enhanced amplicon yield relative to the control, and were greatest when method 3 was employed i.e., the method incorporating a 70°C and 10 minutes of vortexing. In contrast, amplicon concentrations from DNA extracted from cheese were greatest when the unaltered, standard method was employed.

Given that qPCR provides a more rapid means of detecting pathogens in food, the success with which DNA extracted using the PowerFood™ Microbial DNA could be amplified by qPCR was assessed. To facilitate this, standard curves were constructed using a range of different concentrations, between 10^1 and 10^8 gene copies μl^{-1} of *hlyA* and *invA* from *Listeria monocytogenes* and *Salmonella typhimurium*, respectively (Figure 2A and 2B). The efficiency of the constructs were 1.94 and 1.81, respectively. This is a critical parameter in validating the standard curve construct, it was determined by preparing a minimum 5-log dilution series. These values provide good confidence in the accuracy and sensitivity of the method (Larionov et al., 2005). A single product peak at ~77°C for the *hlyA* product and ~79°C for the *invA* product was observed representing the specific melting temperature (T_m) (Figure 2C and 2D respectively). QPCR of DNA extracts from spiked milk accurately detected and quantified the pathogens when present at concentrations of 10^7, 10^5, 10^3 and 10^1 cfu ml^{-1} (Figure 3A and 3B), thereby establishing that DNA extracted from milk using the PowerFood™ Microbial DNA Isolation kit can also be successfully employed for subsequent real-time PCR amplification.

4. Discussion

DNA-based molecular analysis of an environment requires the efficient extraction of DNA from that environment. Here seven methods were assessed to compare their relative success with respect to the extraction of DNA from raw milk and raw milk cheese, as well as their ability to extract DNA from both Gram-positive and Gram-negative microorganisms. More specifically these were examined to assess their relative ability to extract DNA at a high concentration and facilitate subsequent PCR reactions, with the latter depending on the successful removal of inhibitors. While all of the methods were highly successful with respect to the extraction of DNA from cheese, the extraction of DNA from milk varied more dramatically from one approach to another.

The QIAamp® DNA stool mini kit, which has proven success in extracting DNA from faecal samples (Li et al., 2003), on one previous occasion provided poor DNA yields from fresh whole milk and a cow's milk cheese as well as butter, cream and yoghurt (Pirondini et al., 2010). Here, we tested this kit with an additional modification, suggested to improve its use in food products, yielding efficient nucleic acid extraction from cheese; however the yield from milk was poor resulting in poor PCR amplification, thus suggesting the modification is not suitable for all food matrices. Similarly, the Chemagic Food Basic kit and the Wizard® Magnetic DNA Purification System for Food both recovered DNA from cheese that was readily amplified, the DNA extracted by these kits from raw milk was not sufficient. It was previously noted that the use of the Chemagic Food Basic kit to extract DNA from fermented cereals also resulted in a poor yield and a low number of bands from subsequent molecular fingerprinting (Abriouel et al., 2008) and that the Wizard® Magnetic DNA Purification System for Food is not ideally suited to the extraction of DNA from pasteurised milk but was more efficient when used for extractions from vegetable matrices rich in polysaccharides and polyphenolics (Di Pinto et al., 2007). Both the Chemagic Food Basic kit and the Wizard® Magnetic DNA Purification System for Food are based on mobile solid-phase, magnetic-bead DNA separation. The inefficient extraction of DNA by these kits suggests that this technology is not as efficient as other solid-phase extraction methods,

such as column-based methods. The ‘guanidine thiocyanate’ method, although previously employed in studies where the extraction of DNA from milk was successful (Callon et al., 2007, Delbes et al., 2007), was not among the more efficient methods employed here as DNA yields were quite low. Here, we also determined that although these four approaches led to successful detection of pathogens spiked into milk, they were less efficient when pathogen levels were low.

Three methods were found to be particularly effective at extracting both total genomic DNA, as well as DNA from the representative Gram positive and Gram negative pathogens. These were the Milk Bacterial Isolation kit, PowerFood™ Microbial DNA Isolation kit and the ‘Lytic’ method. The Milk Bacterial Isolation kit, which has previous success for extraction of total bacteria from milk (Callon et al., 2007, Delbes et al., 2007), in this instance also, extracted high quality DNA from raw milk. Although designed specifically for milk, we also highlight the efficiency of this kit to extracted DNA from cheese and thus it could potentially be applied to other dairy products. The PowerFood™ Microbial DNA Isolation kit provided highly pure and concentrated DNA from milk and cheese, which in return provided very concentrated PCR amplicons. Both of these methods are based on column extraction which has proven success in extraction of nucleic acids. Finally, the ‘Lytic’ method provided the highest DNA return from cheese and generated the most concentrated amplicons from DNA extracted from either milk or cheese. The inclusion of enzymes has shown previous success in DNA extraction from dairy environments (Dolci et al., 2008, O'Mahony and Hill, 2004, Parayre et al., 2007). Based on the performance of all of the methods with respect to DNA yield, purity and PCR amplification, the PowerFood™ Microbial DNA Isolation kit and ‘Lytic’ method were deemed to be the most successful with regard to the extraction of DNA from raw milk and raw milk cheese. The final decision as to which method should be selected for further optimisation was made by considering the duration of the assays and labour intensity required. Notably, with respect to the ‘Lytic’ method, all reagents had to be prepared in advance and a number of incubation periods, including an over-night incubation step, resulted in a completion time of approximately 20 hours. The PowerFood™ Microbial DNA Isolation kit, a commercial kit, came

in a ready-to-use form and was completed in approximately one *h*. Thus, as a consequence of its rapidity, the PowerFood™ Microbial Isolation kit was deemed the most attractive extraction option and was subjected to further investigation. To determine if its yields could be further improved upon, a number of modifications were tested. These did not improve yields from cheese, which were already quite high, but milk extractions were significantly improved (p=0.005). This improvement was most notable after heating of the sample at 70°C for 10 minutes.

To further assess the suitability of the PowerFood™ Microbial Isolation kit with respect to the detection and quantification of pathogens in milk, milk spiked with *L. monocytogenes* and *S. typhimurium* was employed. Notably these species are recognised worldwide as the leading causes of foodborne illness and are of major concern, not only to the dairy industry, but to the food industry as a whole (Nyachuba, 2010). While conventional PCR methods can determine the presence of a bacterium, these protocols are being replaced by more convenient and rapid real-time PCR assays, allowing the detection and accurate quantification of microbes in a matter of hours. However, as with other DNA-based assays, the success of real-time PCR is dependent on the success with which template DNA can be extracted. Here we have shown the ability of the PowerFood™ Microbial DNA Isolation kit to facilitate the detection of these foodborne pathogens at levels as low as 10^1 cfu ml^{-1}.

5. Conclusion

In conclusion, it was determined that of the seven methods, the PowerFood™ Microbial DNA Isolation kit was best suited to the extraction of total DNA from raw milk and raw milk cheese in that it rapidly generated highly concentrated DNA, which was very pure and which served well as a template for subsequent PCR amplifications. We also established that kit efficiently extracted DNA from representative Gram-positive and Gram-negative pathogens and facilitated subsequent amplification of targets by conventional and real-time PCR. To our knowledge this is the first report of the PowerFood™ Microbial DNA Isolation kit being used to extract DNA from dairy products.

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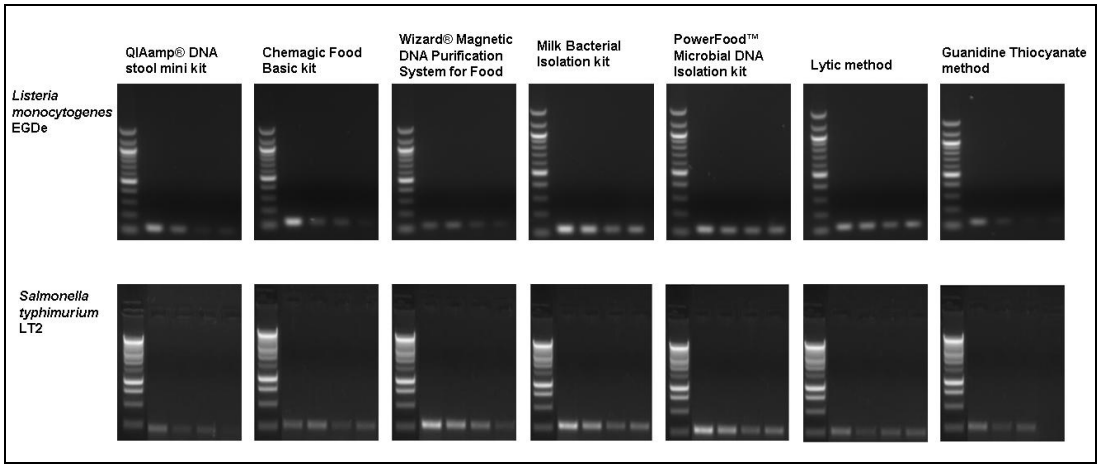

Figure 2: The ability of the seven DNA extraction methods to extract DNA from Gram positive *Listeria monocytogenes* EGDe and Gram negative *Salmonella typhimurium* LT2, suitable for downstream PCR application, as revealed by gel electrophoresis imaging. First lane in each image represents the molecular weight marker, 100bp, followed by PCR amplification products of DNA extracts from milk spiked with 10^7, 10^5, 10^3, 10^1 cfu ml^{-1} respectively.

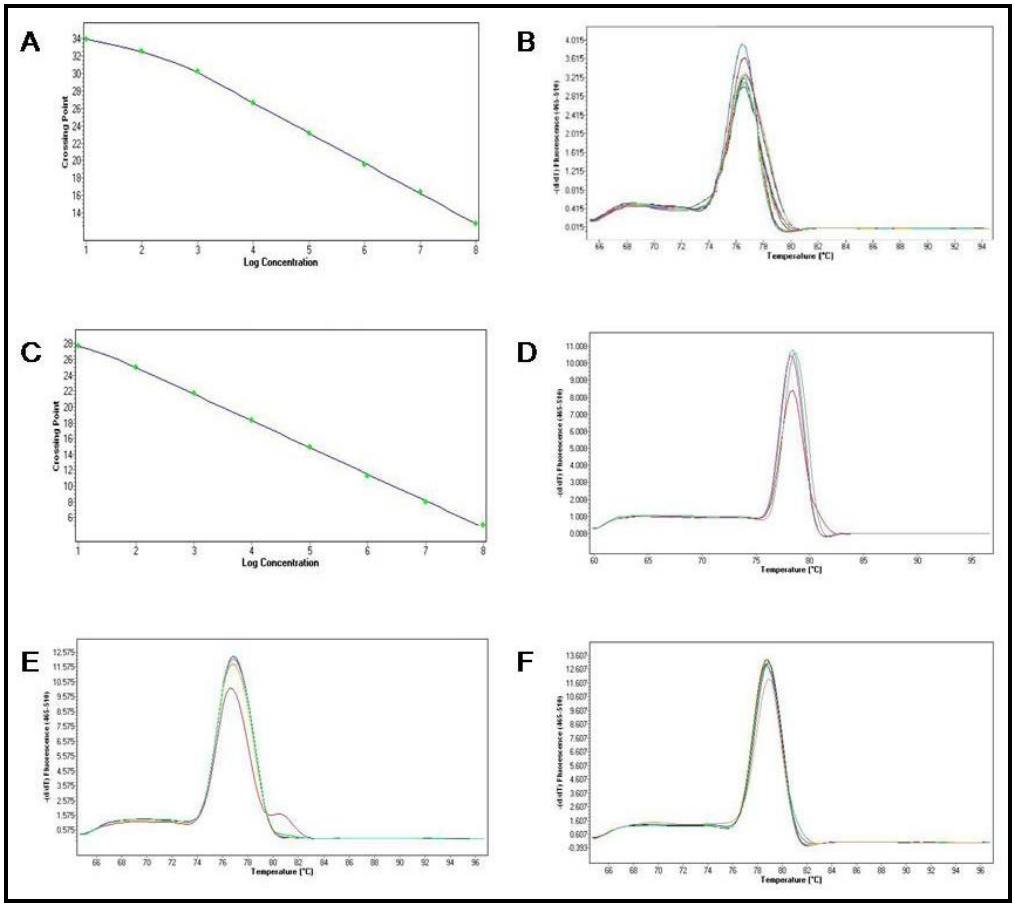

Figure 3: Real-time PCR standard curve construction, A=Standard curve of *Listeria monocytogenes hlyA* gene, efficiency of 1.94; B=Melting curve analysis of standard curve product of *hlyA* gene; C=Standard curve of *Salmonella typhimurium invA* gene, efficiency of 1.81; D=Melting curve analysis of standard curve product of *invA* gene. Melting curve of real-time PCR analysis of DNA extracted, by PowerFood^{TM} Microbial DNA Isolation kit, from raw milk spiked with 10^7, 10^5, 10^3, 10^1 cfu ml^{-1} of E=*Listeria monocytogenes* EGDe and F=*Salmonella typhimurium* LT2.

Table 7: Brief description of the principal of each extraction methods

Method	Extraction Type	Source	Principal
Modified QIAamp® DNA stool mini kit (Qiagen Ltd.)	Solid-phase/ column extraction	Commercial	Cell lyses using chaotrophic agents, detergents, proteinase K and heating, uses an exclusive adsorption resin to remove impurities. DNA purification uses a silica-gel membrane.
Chemagic Food Basic kit (Chemagen Bioploymer-Technologie)	Mobile solid-phase/ magnetic bead extraction	Commercial	Cell lyses using chaotrophic agents and RNAse A. Magnetic beads as solid-phase for binding target DNA.
Wizard® Magnetic DNA Isolation kit (Promega Inc.)	Mobile solid-phase/ magnetic bead extraction	Commercial	Cell lyses using chaotrophic agents and RNAse A. DNA bound and purified using magnetic beads as solid phase support.
Milk Bacterial DNA Isolation kit (Norgen Biotek Corp.)	Solid-phase/ column extraction	Commercial	Cell lyses using chaotrophic agents and enzymes. DNA binding and purification using unique resin separation matrix and spin column chromatography.
PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc.)	Solid-phase/ column extraction	Commercial	Cell lyses based on chaotrophic agents, mechanical lyses and inhibitor removal technology. DNA binding is based on silica membrane spin column.
Lytic method (In-house developed)	Liquid-liquid extraction	Non-commercial	Cell lyses using chaotrophic agents, enzymes and mechanical lyses. DNA extraction using phenol-chloroform and ethanol purification
Guanidine Thiocyanate method (Duthoit et al., 2003)	Liquid-liquid extraction	Non-commercial	Cell lyses using detergents, chaotrophic agents, mechanical lysis plus heat. DNA extraction using phenol-chloroform and ethanol purification

Table 2: Details of species-specific primers used for the amplification of Gram-negative and Gram-positive bacterial DNA extracts from spiked milk study

Strain	Target	Primer	Sequence (5' to 3')	Annealing temperature	Size	Reference
Listeria monocytogenes EGDe	Listeriolysin O gene	*hlyA* Forward	TGCAAGTCCTAAGACGCCA	60°C	113bp	(Nogva et al., 2000)
		hlyA Reverse	CACTGCATCTCCGTGGTACTAA			
Salmonella typhimurium LT2	Invasion gene	*invA* Forward	TCGTCATTCCATTACCTACC	55°C	119bp	(Nam et al., 2005)
		invA Reverse	AAACGTTGAAAAACTGAGGA			

Table 3: Comparison of seven extraction methods assessed

	Extraction Method	DNA Yield[A] ng ml^{-1} or g^{-1}	DNA Purity (A260/280nm)	PCR Yield[B] ng rxn^{-1}
Milk	QIAamp® DNA stool mini kit	382.34 ± 54.8[6]	1.78[3]	947.22 ± 11.55[5]
	Chemagic Food Basic kit	425.61 ± 73.3[5]	1.27[5]	247.31 ± 2.99[6]
	Wizard® Magnetic DNA Purification System for Food	676.42 ± 91.11[4]	1.23[6]	974.48 ± 11.97[4]
	Milk Bacterial Isolation kit	835.96 ± 57.29[2]	1.56[4]	2518.03 ± 188.56[3]
	PowerFood™ Microbial DNA Isolation kit	909.53 ± 6.0[1]	1.85[2]	5132.86 ± 77.47[2]
	Lytic method	776.42 ± 25.5[3]	1.23[6]	5143.43 ± 62.97[1]
	Guanidine Thiocyanate method	132.28 ± 24.39[7]	1.92[1]	69.82 ± 1.34[7]
Cheese	QIAamp® DNA stool mini kit	2155.69 ± 55.89 [4]	1.39[6]	7060.53 ± 50.71[4]
	Chemagic Food Basic kit	1624.05 ± 95.1[5]	2.30[1] *	3648.06 ± 35.95[5]
	Wizard® Magnetic DNA Purification System for Food	1308.91 ± 32.82[6]	1.01[7]	3092.16 ± 54.76[7]
	Milk Bacterial Isolation kit	5283.10 ± 47.42[3]	1.51[5]	7068.13 ± 36.66[3]
	PowerFood™ Microbial DNA Isolation kit	6756.14 ± 16.47[2]	1.92[2]	7303.86 ± 103.86[2]
	Lytic method	7147.04 ± 10.85[1]	1.63[4]	8866.3 ± 50.7[1]
	Guanidine Thiocyanate method	336.54 ± 80.68[7]	1.85[3]	3642.23 ± 257.95[6]

Results represent the mean ± standard error calculated from triplicate assessment in each case. Numbers 1-7 indicate the relative success of each method (i.e. 1=best, 7=worst) for each of the three assessment criteria. A: Samples are standardised as DNA yield per ml of milk or per g of cheese; B: PCR yield is standardised according to PCR template volume of 5 µl.

* The high purity ratio indicates an excess of reagents from the extraction method which may interfere with downstream application of DNA.

Table 4: Results from attempts at improving the yield of DNA extracted using the PowerFood™ Microbial DNA Isolation kit

Sample	Method	DNA Yield[A] ng ml^{-1} or g^{-1}	DNA Purity (A260/280nm)	PCR Yield[B] ng rxn^{-1}
Milk	C	896.65 ± 52.70[5]	1.72[5]	4997.06 ± 53.42[5]
	1*	2713.96 ± 40.77[3]	1.83[3]	5403.7 ± 69.82[4]
	2*	3255.1 ± 20.3[2]	1.79[4]	6568.6 ± 144.39[2]
	3*	3471.53 ± 119.15[1]	2.00[1]	6934.13 ± 27.28[1]
	4*	439.56 ± 32.01[6]	1.84[1]	776.6 ± 33.08[6]
	5*	2316.36 ± 38.94[4]	1.84[1]	5596.3 ± 92.7[3]
Cheese	C	9062 ± 110.95[2]	1.97[1]	11224.87 ± 25.72[2]
	1	8162.83 ± 234.23[5]	1.80[4]	9606.33 ± 90.74[5]
	2	8492.53 ± 208.23[4]	1.79[5]	9899.26 ± 46.04[4]
	3	9079.2 ± 63.13[1]	1.79[5]	10139.33 ± 79.68[3]
	4*	4673 ± 89.92[6]	1.83[3]	10009.4 ± 35.11[1]
	5	8893.76 ± 172.45[3]	1.89[2]	9467.13 ± 126.38[6]

Results represent the mean ± standard error calculated from triplicate assessment in each case.

C: control i.e. kit without additional steps; 1: heating sample to 65°C for 10 min; 2: heating samples to 65°C for 10 min with occasional vortexing; 3: heating samples to 75°C for 10 min; 4: ethanol precipitation on eluted DNA; 5: incorporation of enzymes with solution PF1, i.e. 50 µg ml^{-1} lysozyme and 100U mutanolysin incubated at 37°C for 1 h followed by 250 µg ml^{-1} proteinase K for 1 h at 55°C.

Numbers 1-6 indicate the relative success of each method (i.e. 1=best, 6=worst) for each of the three assessment criteria. A: Samples are standardised as DNA yield per ml of milk or per g of cheese; B: PCR yield is standardised according to PCR template volume of 5 µl.

* A statistical significance was observed between the control sample and the additional steps to improve DNA extraction $p < 0.05$.

Chapter II

High-throughput sequencing detects subpopulations of bacteria not previously associated with artisanal cheeses

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Abstract

Here, high-throughput sequencing was employed to reveal the highly diverse bacterial populations present in 62 Irish artisanal cheeses and, in some cases, associated cheese rinds. Using this approach, we revealed the presence of several genera not previously associated with cheese, including *Faecalibacterium, Prevotella* and *Helcococcus* and, for the first time, detected the presence of *Arthrobacter* and *Brachybacterium* in goat's milk cheese. Our analysis confirmed many previously observed patterns such as the dominance of typical cheese bacteria, the fact that the microbiota of raw and pasteurised milk cheeses differ and that the level of cheese maturation has a significant influence on *Lactobacillus* populations. It was also noted that cheeses containing adjunct ingredients had lower proportions of *Lactococcus* species. It is thus apparent that high-throughput sequencing-based investigations can provide a valuable insight into the microbial populations of artisanal foods.

1. Introduction

High-throughput sequencing has revolutionised the field of microbial ecology, allowing for a more accurate identification of microbial taxa, including those which are difficult to culture and/or are present in low abundance (Sogin et al., 2006). These technologies have provided a detailed insight into the microbial composition of a wide variety of different ecosystems including sea (Sogin et al., 2006), soil (Roesch et al., 2007) and gut environments (Andersson et al., 2008, Claesson et al., 2009), as well as that of a relatively-small selection of food-associated niches (Dobson et al., 2011, Masoud et al., 2011, Roh et al., 2010). One group of complex microbial environments not assessed, to date, in this way are artisanal cheeses. The complex, fermentation-based nature of cheese means that the microbiota of different cheeses varies considerably. Many of these microbes are also hugely influential with respect to the textural and organoleptic properties of a cheese (Marilley and Casey, 2004). Thus, unsurprisingly there have been considerable efforts made to characterise the microbial populations of cheeses. Traditional culture-independent molecular methods, most frequently the analysis of 16S rRNA genes through denaturing or temporal temperature gradient gel electrophoresis (DGGE/TTGE) (Ercolini, 2004, Ogier et al., 2004), single stranded conformation polymorphisms (SSCP) (Callon et al., 2007) and/or Sanger sequencing (Duthoit et al., 2003), have improved our understanding of cheese microbial population (Quigley et al., 2011). However, we anticipated that the application of high-throughput sequencing could provide an even more detailed understanding of the microbial composition of cheese. Thus we have applied this technology to investigate the microbiota of 62 soft, semi-hard and hard artisanal cheeses, which have been manufactured from unpasteurised or pasteurised cow's, goat's and sheep's milk and of 11 associated naturally developed or smear-ripened rinds.

2. Materials and Methods

2.1 Cheese Collection and Nucleic Acid Extraction

A total of 62 handmade cheeses, 18 soft cheeses, 31 semi-hard cheeses and 13 hard cheeses, manufactured from unpasteurised or pasteurised cows', goat's or sheep's milk, were obtained from artisanal cheese producers and farmer's markets throughout Ireland (Table S1). To facilitate the culture independent analysis of the bacterial composition of these cheeses, their associated rinds, naturally developed or smear-ripened cheese rinds, were also analysed. 1 g of cheese or 1 g of cheese rind (Callon et al., 2006, Coppola et al., 2001, Delbes et al., 2007, Duthoit et al., 2003, Ercolini et al., 2003) was combined with 9 ml 2% tri-sodium citrate and homogenised before DNA was extracted using the PowerFood^{TM} Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) (Quigley et al., 2012).

2.2 PCR amplification of the microbial community 16S rRNA gene

The DNA extracts were used as a template for PCR amplification according to Quigley et al (Quigley et al., 2012). Here, universal 16S primers targeting the V4 region (239 nt long) predicted to bind to 94.6% of all 16S genes were incoporated i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The primers incorporated a proprietary 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5' end to allow emulsion-based clonal amplification for the 454-pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adapatmer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. The PCR reaction contained 25 µl GoTaq Green Master Mix (Promega), 1 µl of each primer (10 pmol), 5 µl DNA template and nuclease free dsH_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, UK). The amplification programme consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles; denaturation at

94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. Amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, United Kingdom). The quantity of DNA extracted was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen, USA) used in accordance with the manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, USA).

2.3 High-throughput sequencing and bioinformatics analysis

The 16S rRNA V4 amplicons were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) according to Roche 454 protocols. Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (Cole et al., 2009). Sequences not passing the FLX quality controls were discarded, the 454 specific portion of the primer were trimmed, the raw sequences were sorted according to tag sequences and reads with low quality scores (quality scores below 40) and short length (less than 150 bp for the 16S rRNA V4 region) were removed as were reads that did not have exact matches with respect to primer sequence. Statistical analysis, to measure the sequencing diversity included Chao1 richness, Shannon diversity and Good's Coverage, as well as, monitoring sequencing abundance using rarefaction analysis, were performed using the MOTHUR package (Schloss et al., 2009). Principal Co-ordinate Analysis, measuring dissimilarities at phylogenetic distances based on Weighted Unifrac was performed using the QIIME suite of programs (Caporaso et al., 2010). Trimmed fasta sequences were assessed by BLAST analysis (Altschul et al., 1990) against a previously published 16S-specific database (Urich et al., 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarisation (absolute cut-off: BLAST bit-score 86, relative cut-off: 10% of the top hit) (Urich et al., 2008). Statistical significance was determined by

the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) using the Minitab® statistical package.

3. Results

3.1 Sequencing and Bioinformatic Analysis

DNA was extracted from a 1g sample size from 62 cheeses and from the rinds of 11 of the cheeses (Table S1). Following total genomic DNA extraction, amplicons of the V4 16S rRNA gene were generated and a total of 116,238 pyrosequencing reads were obtained through 454 sequencing, corresponding to 32,322, 48,388 and 18,340 reads from soft, semi-hard and hard cheeses, respectively, and 17,188 reads corresponding to cheese rinds. Diversity, richness and coverage estimations were calculated for each data set (Table 1; individual sample diversity are presented in Table S2). The Chao1 estimator of species richness indicates good sample richness throughout. The Shannon diversity index, a measurement of overall diversity, indicates a diverse microbiota, while Good's coverage, an estimator of completeness of sampling, highlights good overall sampling with levels of 89-95%. Rarefaction curve analysis, which assesses species richness from the results of sampling, show all samples approaching parallel with the x-axis, revealing that the overall bacterial diversity is well represented (Fig. 1). Principal Co-ordinate Analysis (PCoA), which clusters the communities according to different parameters, in this case cheese type, animal source of milk or whether the milk was pasteurised or not, was examined according to weighted UniFrac distances (Fig. 2). Regardless of the community parameters, there is no definitive split in the microbiota of the cheese. However, the most extreme outliers generally tend to be cheese rinds from cows milk cheeses. No statistical differences were found in OTU at phylum level, however a number of statistical differences were determined at genus level. The gene sequence information has been prepared in a MiXS-MIMARKS metatable (Yilmaz et al., 2011), shown in Supplementary data (Table S3 - available online only).

3.2 The microbial composition of artisanal cheese as revealed by pyrosequencing

In silico analysis of high-throughput sequence data revealed microorganisms corresponding to five phyla in the soft, semi-hard and hard cheeses (Table 2).

These were representatives of four bacterial phyla i.e. the Firmicutes, Proteobacteria, Bacteroidetes and Acintobacteria. Surprisingly, a fifth phylum detected was the fungal phylum Ascomycota. The latter were detected occasionally throughout the cheese samples at a subdominant level, i.e. 0.37-0.50%, and at genus level corresponded almost exclusively with *Penicillium*. Further examination of the *Penicillium* sequence established that it corresponds to that of the mitochondrial 16S rRNA gene of *Penicillium*. Of the four bacterial phyla, Firmicutes dominated in the three cheese types, corresponding to 96%, 95% and 91% of the reads from the soft, semi-hard and hard cheeses, respectively. Proteobacteria (0.91, 3.31 and 1.79%), Bacteroidetes (0.27, 0.25 and 0.21%) and Actinobacteria (1.22, 0.12 and 4.45%) were detected at varying levels throughout (Table 2).

The bacteria present corresponded to 21 different genera (Fig. 3; Table 2), with *Lactococcus* dominating. At the depth of analysis carried out, a total of eight genera were found to be common in all three cheese types (soft, semi-hard and hard). In addition to *Lactococcus* (89.93, 84.45 and 49.56%), *Lactobacillus* (0.65, 7.30 and 17.8%), *Leuconostoc* (1.79, 0.51 and 1.8%), *Pseudomonas* (0.11, 0.03 and 0.49%), *Psychrobacter* (0.58, 0.02 and 0.53%), *Staphylococcus* (0.06, 0.17 and 0.73%), *Arthrobacter* (0.28, 0.08 and 1.1%) and *Faecalibacterium* (0.02, 0.08 and 0.05%) were identified also. Statistically significant differences was observed in the levels of *Lactococcus* (*P*=0.031) and *Lactobacillus* (*P*=0.010), with the level of lactococci increasing and the level of lactobacilli decreasing between soft, semi-hard and hard cheese types (Fig. 3). *Vibrio* were found in soft cheese only (0.02%), *Helcococcus* (0.07%), *Halomonas* (0.25%) and *Streptococcus* (0.04%) were found in the semi-hard cheeses only, while *Enterococcus* (0.1%), *Tetragenococcus* (0.05%) and *Clostridium* (0.06%) were found in hard cheeses only. Three genera were shared between soft and semi-hard cheeses. These were *Pseudoalteromonas* (0.06 and 0.03%), *Pediococcus* (0.03% and 0.27%) and *Bifidobacterium* (0.02% and 0.03%). *Brevibacterium* (0.81% and 2.10%) was the only genus shared between soft and hard cheeses and *Prevotella* (0.15% and 0.34%) was the only genus common to semi-hard and hard cheeses.

Some interesting observations were made regarding the influence of the animal source of milk and pasteurisation on the microbial populations present in the resultant cheeses. It was noted that cow's milk cheese contained 21 different bacterial genera (Table 2) whereas goat's milk contained only 8 different bacterial genera and only two bacterial genera, *Lactococcus* and *Lactobacillus*, were detected in sheep's milk cheese. Also, by comparing the bacterial genera present in artisanal cheeses manufactured from unpasteurised, relative to those made from pasteurised, milk, it was apparent that *Halomonas, Helcococcus, Streptococcus, Enterococcus* and *Tetragenococcus* were detected in raw milk cheeses only and that *Clostridium* and *Vibrio* were detected from pasteurised milk cheeses only. A significant difference was noted in the levels of *Lactococcus* (p=0.025) and *Lactobacillus* (p=0.002) between unpasteurised and pasteurised milk cheeses. Further comparisons provided some interesting findings. Cheeses S2 and S3 were produced with milk from the same herd using similar protocols, but differed in that S3 is a feta style cheese and thus has a higher salt content which may explain the absence of *Leuconostoc* and *Pseudomonas* from this cheeses. Similarly, S7 and H9 are produced in the same farmhouse but differ with respect to the level of maturation with associated differences in the proportions of *Lactobacillus* (0.65%, soft cheese; 17.8%, hard cheese). This is reflective of the aforementioned overall greater number of lactobacilli in hard relative to semi-hard and, in turn, soft cheeses. This pattern is also apparent when SH1 and H3-H6, all from the same producers, are compared. In addition, a specific comparison of H4 and H5 is also interesting as these cheeses differ solely on the basis that H5 contains an adjunct ingredient, fenugreek seeds, the presence of which coincides with a reduction in the proportion of lactococci (from 61 to 2%) and increase in lactobacilli (from 34 to 95%). The inclusion of herbs, spices or seaweed was also found to coincide with reduced proportions of lactococci in SH8 relative to SH10-11 and in SH28 relative to SH26, respectively.

3.3 Revealing the microbial composition of the rind of artisanal cheeses

We again used high-throughput sequencing to analyse the microbiota of 11 of the artisanal cheeses rinds (R1-11) (Table S1; Fig. 3). These included

smear/wash ripened rinds, i.e. R1, R7, R8 and R9, naturally developed rinds, i.e. R2-R6 and R11, and one mould ripened rind, R10. *In silico* analysis of sequence data revealed the presence of 19 different genera (Fig. 3; Table 2). While some of these genera, including *Lactococcus, Leuconostoc and Lactobacillus*, corresponded to those also detected in the cheese core, a selection were identified in cheese rinds only. These included *Corynebacterium* (1.2%), *Facklamia* (0.60%), *Flavobacterium* (0.19%) and *Cronobacter* (0.05%). While lactococci remained the most common genus in cheese rinds, the relative proportions of this genus were significantly lower in the rind than in the core. Generally, smear/wash-ripened rinds had particularly low levels of lactococci (1.9-4.8%), while naturally developed rinds had levels of lactococci of up to 98%. It was also apparent that *Psychrobacter* and *Brevibacterium* represented a considerable proportion, i.e. 0.29-57% and 0.67-54.6%, respectively, or ~10% on average, of the total population. The other genera detected, i.e. *Leuconostoc, Lactobacillus, Pseudomonas, Psychrobacter, Pseudoalteromonas, Brachybacterium, Prevotella, Arthrobacter, Streptococcus, Tetragenococcus* and *Facklamia,* corresponded to between 0.03 and 4.14% of reads (Table 2). *Brevibacterium* and *Brachybacterium* had significant differences in the levels present in the rinds of soft, semi-hard and hard cheeses compared to cheese core, p=0.040 and p=0.014, respectively. *Penicillium* was also detected in the cheese rind and in higher proportions than were detected in the cheese core. Finally, we also detected the presence of *Prevotella*, a genera which has previously not been detected in cheese or cheese rinds and noted that *Vibrio* were only identified in rinds of the smear/wash developed variety (p=0.009).

4. Discussion

Here, pyrosequencing based 16S rRNA profiling has provided a detailed insight into the complex microbiota of artisanal cheeses. Its use effectively revealed the presence of a number of taxa not previously associated with specific cheese types or, indeed, of any cheeses. Among those identified for the first time were the genera *Prevotella* and *Faecalibacterium*. *Prevotella* are Gram-negative bacteria from the phylum Bacteroidetes that thrive in anaerobic environments. They are commensals of the rumen and hind gut in cattle and sheep but can also be the cause of periodontal disease as well as other human infections. Members of the genus *Faecalibacterium* are strict anaerobes and have been shown to produce butyrate, D-lactate and formate, as well as utilise acetate (Duncan et al., 2002). While butyrate can contribute positively to cheese development, in high levels this product can induce the late-blowing defect in cheese (Cocolin et al., 2004). D-lactate and acetate are also produced during the development of cheese (Fox, 1999). Further investigations will be required to determine if, at the levels present in cheese, these microbes contribute flavour in a significant way. A third genus which is typically associated with anaerobic gastrointestinal environments, i.e. *Helcococcus*, was also detected but only one cheese, a semi-hard cheese made from unpasteurised cow's milk. *Helcococcus* have been associated with clinical problems in humans (Collins et al., 2004), in cows (Kutzer et al., 2008), sheep (Zhang et al., 2009) and horses (Rothschild et al., 2004) and thus, in this instance, may reflect the sourcing of contaminated milk from an infected animal. Given that, in this study, these insights were gained through the analysis of merely a 1 g sample per cheese, it may be that further investigations of even larger sample sizes and at a greater depth of sequencing will uncover additional genera not previously associated with cheese. Nevertheless, the detection of these anaerobes reveals that the microbiota of cheese is more diverse than previously appreciated, thus further highlighting the benefits of high-throughput sequencing investigations.

We also detected a number of genera not previously associated with specific cheese types. Here we noted that *Arthrobacter* and *Brachybacterium* were detected for the first time in goat's cheese, these are commonly detected on

cheese surface but not, to our knowledge, in the core of goat's cheese. The presence of *Pseudoalteromonas* in soft and semi-hard cow's milk cheeses, as well as cheese rinds was also unexpected. *Pseudoalteromonas* species are usually regarded as marine bacteria (Bozal et al., 2003) and have been detected on the surface of smear-ripened cheese on only one previous occasion (Feurer et al., 2004a). This is the first instance upon which this genus has been detected in a cheese core.

In addition to identifying taxa not previously associated with cheeses or specific cheese types, there were a number of other interesting observations. It was noted that milk source impacted on the number of genera detected, i.e. 21 genera from cow's milk cheese, 8 from goat's milk cheese and 2 from sheep's milk cheese, although this observation may be influenced by differences in the number of samples within each group. Notably, a number of studies have previously established that milk source can influence the type and number of microbes present in a cheese (Coppola et al., 2001, Randazzo et al., 2006). Previous studies have also highlighted the dramatic impact of milk pasteurisation on the microbiota of resultant cheeses (Bonetta et al., 2008, Coppola et al., 2001, Duthoit et al., 2005). Here, through the use of high-throughput sequencing, we also observed differences in cheeses produced from raw and pasteurised milk (Table 2) in that, for example, significant differences in levels of *Lactococcus* and *Lactobacillus* were apparent when these cheese types were compared. For further studies to investigate these differences, it would be interesting to focus on RNA (and thus cDNA), or to employ stains that inactivate DNA from cells which have been killed by the temperature treatment to determine if such approaches provide different results.

Several previous studies have employed other technologies to investigate the impact of salt (Fox et al., 2004), ripening (Martin-Platero et al., 2009) and additional ingredients (Ankri and Mirelman, 1999, Dash et al., 2011, Tajkarimi et al., 2010) on the microbiota of cheese. Here we noted that neither *Leuconostoc* nor *Pseudomonas* were detected in cheeses with a high salt content, significantly increased *Lactobacillus* populations were detected in cheeses from the same farmhouse but which had been ripened to varying

degrees and observed that the inclusion of adjunct ingredients such as herbs, spices or seaweed did impact on microbial composition.

As a consequence of its exposure to the external environment and, in some cases, steps taken during the manufacturing process, the microbiota of the rind of cheese will frequently differ dramatically from that of the rest of the cheese (Feurer et al., 2004b). This presumably reflects the exposure of the cheese rinds to the environment. Many of the bacterial genera detected are commonly identified in cheese rinds and, indeed, *Corynebacterium, Arthrobacter, Brevibacterium* and *Halomonas* have previously been identified on the surface of Irish artisanal cheeses (Mounier et al., 2005). The high proportions of *Psychrobacter* and *Brevibacterium* in the cheese rinds studied here was particularly notable. *Brevibacterium* is known to be involved in the development of cheese rind flavours and smear rind colour (Krubasik and Sandmann, 2000). Although *Psychrobacter* is frequently detected on cheese surfaces, its specific role is unclear. It may contribute to flavour given the ability of strains from this genus to produce branched-chain aldehydes, alcohols and esters (Deetae et al., 2007). The impact of the presence of such high proportions of these bacteria on the cheese surface will require further investigation. The previously unreported or rare phenomenon of *Prevotella*, *Facklamia* (Roth et al., 2011) or *Vibrio* being detected on cheese rinds further highlights the benefits of employing high-throughput sequencing to investigate these populations.

5. Conclusion

Thus, in conclusion, we have employed high-throughput sequencing to investigate the microbiota of 62 Irish artisanal cheeses in greater depth than ever before. We have highlighted for the first time the presence of a number of genera previously undetected in cheese, as well as, detecting genera not typically associated with specific cheese types. These analyses also provide an insight into the influence of different factors on the composition of the artisanal cheese microbiota which can now be investigated in greater depth through the study of cheeses prepared in the laboratory.

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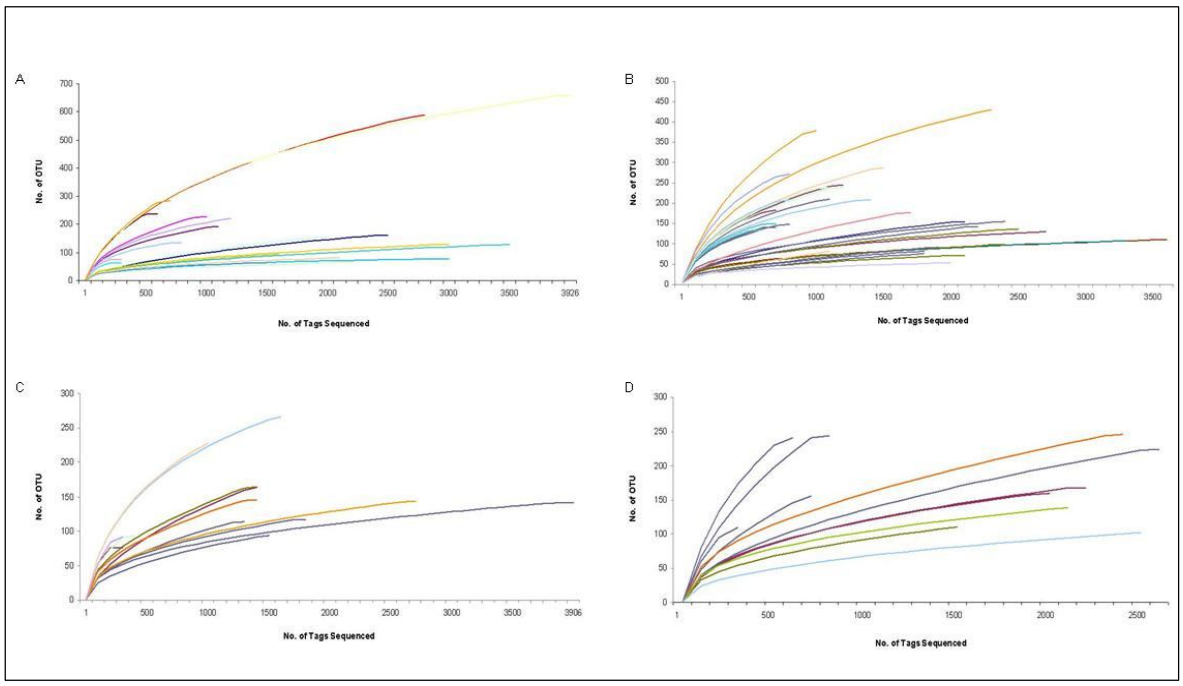

Figure 4: Rarefaction curve of microbial populations from artisanal cheeses and cheese rinds.

Each line represents a cheese sampled and sequenced, A-soft cheeses; B-semi-hard cheeses, C-hard cheeses, D-cheese rinds. The curvature of the line towards the right (or x-axis) shows that a reasonable number of sequenced have been taken, thus sampling is sufficient.

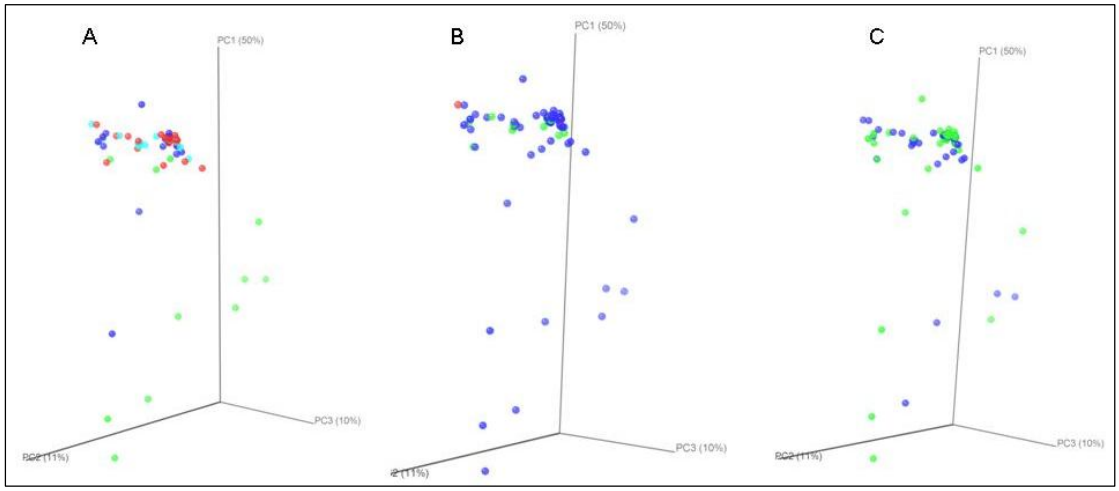

Figure 5: Principal Coordinate Analysis graphs for Weighted UniFrac analysis.

Samples were assessed for different community parameters: A - Cheese Type (soft=light blue, semi-hard=red, hard=dark blue, rind=green); B - Animal Source (cow=dark blue, goat=green, sheep=red); C – Milk (unpasteurised=green, pasteurised=blue).

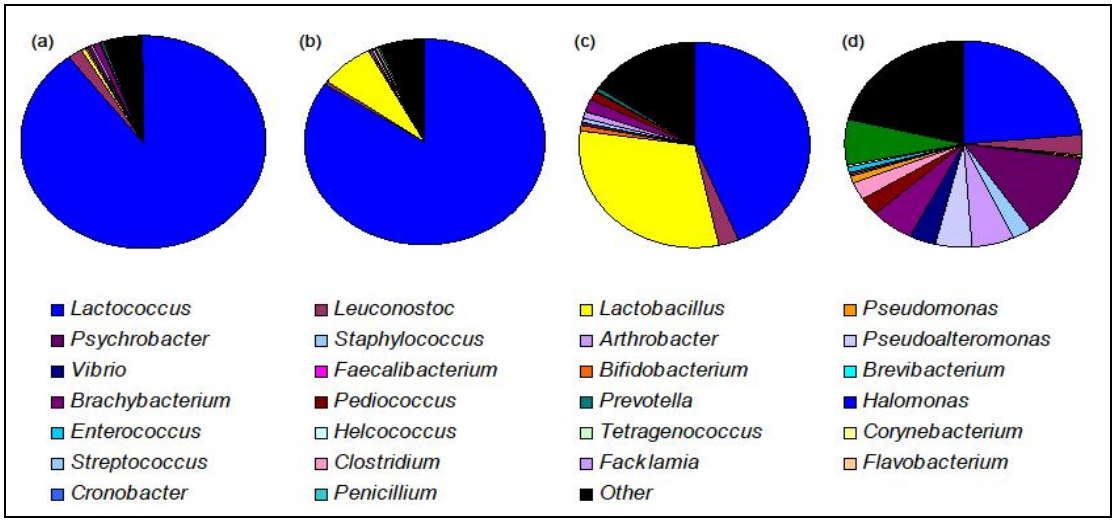

Figure 3: Assignment of cheese microbiota at genus level, according to MEGAN, a= soft cheese; b = semi-hard cheese; c = hard cheese; d = cheese rinds.

Table 1: Average statistical analysis of artisanal cheese, at two different similarity levels, determining sequencing richness, diversity and coverage as analysed by MOTHUR software. The analyses are separated on the basis of cheese type.

	Cheese Type		
Data Set	*Soft*	*Semi-Hard*	*Hard*
Similarity	97%	97%	97%
Chao1 richness estimation	295	315	254
Shannon index for diversity	3.8	4.3	3.7
Good's coverage	92%	90%	91%

Table 2: Percentage of reads calculated from the total phylum reads for each variable assessed.

	Cheese Type				Animal Source			Milk	
	Soft	**Semi-Hard**	**Hard**	**Rind**	**Cow**	**Goat**	**Sheep**	**Unpasteurised**	**Pasteurised**
PHYLUM									
Proteobacteria	0.91	3.31	1.79	26.99	95.5	89.6	98.6	96.1	93.1
Firmicutes	96.03	95.49	91.41	32.14	0.69	1.4	0	0.74	0.83
Acintobacteria	1.22	0.12	4.45	26.02	1.4	6.8	0	1.9	2.5
Bacteroidetes	0.27	0.25	0.21	5.47	0.19	1.1	0	0.22	0.48
Ascomycota	0.49	0.37	0.50	7.13	0.50	0.89	1.38	0.61	0.60
GENUS									
Lactococcus	89.83	84.45	49.56	25.80	77.2	76.0	98.5	72.8	84.4
Leuconostoc	1.79	0.51	1.80	2.57	1.0	2.2	0	1.2	1.1
Lactobacillus	0.65	7.30	17.80	0.20	8.1	1.0	0.08	11.3	0.82
Pseudomonas	0.11	0.03	0.49	0.14	0.07	0.64	0	0.09	0.25
Psychrobacter	0.58	0.02	0.53	9.92	0.28	0	0	0.21	0.23
Staphylococcus	0.06	0.17	0.73	1.98	0.31	0	0	0.30	0.17
Arthrobacter	0.28	0.08	1.10	4.14	0.39	0.85	0	0.49	0.39
Pseudoalteromonas	0.06	0.03	0	3.80	0.03	0	0	0.03	0.02
Vibrio	0.02	0	0	2.84	0.004	0	0	0	0.008
Faecalibacterium	0.02	0.08	0.05	0	0.07	0	0	0.07	0.04
Bifidobacterium	0.02	0.03	0	0	0.02	0	0	0.02	0.009
Brevibacterium	0.02	0	2.10	9.22	0.57	5.4	0	0.82	1.9
Brachybacterium	0.81	0	1.45	3.56	0.39	0.50	0	0.57	0.17
Pediococcus	0.03	0.27	0	0	0.12	0	0	0.17	0.01
Prevotella	0	0.15	0.34	0.03	0.07	0.048	0	0.10	0.16
Halomonas	0	0.25	0	2.46	0.12	0	0	0.1	0
Enterococcus	0	0	0.10	0	0.02	0	0	0.03	0
Helcococcus	0	0.07	0	0	0.03	0	0	0.05	0
Tetragenococcus	0	0	0.05	0.18	0.01	0	0	0.02	0
Corynebacterium	0	0	0	1.20	0	0	0	0	0
Streptococcus	0	0.04	0	0.24	0.01	0	0	0.02	0
Clostridium	0	0	0.06	0	0.01	0	0	0	0.02
Facklamia	0	0	0	0.60	0	0	0	0	0
Flavobacterium	0	0	0	0.19	0	0	0	0	0
Cronobacter	0	0	0	0.05	0	0	0	0	0
Penicillium	0.49	0.37	0.51	12.96	0.50	0.89	1.38	0.61	0.60
Other	5.16	6.14	23.34	17.91	10.67	12.47	0.04	11.1	9.7

Supplementary Data

Table S1: Classification of cheese and rinds sampled.

Soft Cheese				Semi-Hard Cheese				Hard Cheese				Cheese Rind		
Cheese Type	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**
S1[a]	C	UP	-	SH1	C	UP	-	H1	C	UP	-	R1[a]	C	UP
S2	G	UP	-	SH2	C	UP	-	H2	C	UP	-	R2[b]	C	UP
S3	G	UP	-	SH3	C	UP	Oak Smoked	H3[b]	C	UP	-	R3[c]	C	UP
S4	C	UP	-	SH4	C	UP	Cumin Seed	H4	C	UP	-	R4[d]	C	UP
S5	C	UP	-	SH5	C	UP	Fenugreek	H5	C	UP	Fenugreek	R5[e]	C	UP
S6	G	P	-	SH6	C	UP	Black Pepper	H6[c]	C	UP	-	R6[f]	G	UP
S7	G	P	-	SH7	C	UP	Nettle & Garlic	H7	C	UP	-	R7[g]	C	P
S8	C	P	-	SH8	C	UP	-	H8[d]	C	UP	-	R8[h]	C	P
S9[h]	C	P	-	SH9	C	UP	Peppers	H9[f]	G	UP	-	R9[i]	G	UP
S10[j]	C	P	-	SH10	C	UP	Garlic & Herb	H10	C	P	-	R10[j]	C	P
S11	G	P	-	SH11	C	UP	Cumin seed	H11	C	P	-	R11[k]	S	P
S12	G	P	-	SH12	C	UP	Oak Smoked	H12	G	P	-			
S13	G	P	-	SH13	C	UP	Garlic & Herb	H13	G	P	-			
S14	C	P	-	SH14	C	UP	Pepper & Chive							
S15	C	P	Garlic & Herbs	SH15	C	UP	-							
S16	C	P	Oak Smoked	SH16	C	UP	Seaweed							
S17[g]	C	P	-	SH17	C	UP	Garlic							

Table S1 continued:

Soft Cheese				Semi-Hard Cheese				Hard Cheese				Cheese Rind		
Cheese Type	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**
S18[l]	C	P	-	SH18	C	UP	Nettle & Onion							
				SH19	C	UP	Black Pepper							
				SH20[e]	C	UP	-							
				SH21	C	P	-							
				SH22	C	P	-							
				SH23	C	P	-							
				SH24	C	P	Cumin Seed							
				SH25	C	P	-							
				SH26	C	P	-							
				SH27	C	P	Basil & Garlic							
				SH28	C	P	Seaweed							
				SH29[k]	S	P	-							
				SH30	S	P	-							
				SH31	S	P	-							

Classification according to cheese type is based on cheese texture S=soft, SH=semi-hard, H=hard, and the cheese rind R=rind. Other factors considered are: animal source, according to the type of animal that milk used for cheese manufacture was sourced from C=cow, G=goat or S=sheep; and the milk treatment i.e., UP=unpasteurised or P=pasteurised. The subscript letters a-k indicates the cheese and corresponding rind.

Chapter II

High-throughput sequencing detects subpopulations of bacteria not previously associated with artisanal cheeses

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Abstract

Here, high-throughput sequencing was employed to reveal the highly diverse bacterial populations present in 62 Irish artisanal cheeses and, in some cases, associated cheese rinds. Using this approach, we revealed the presence of several genera not previously associated with cheese, including *Faecalibacterium, Prevotella* and *Helcococcus* and, for the first time, detected the presence of *Arthrobacter* and *Brachybacterium* in goat's milk cheese. Our analysis confirmed many previously observed patterns such as the dominance of typical cheese bacteria, the fact that the microbiota of raw and pasteurised milk cheeses differ and that the level of cheese maturation has a significant influence on *Lactobacillus* populations. It was also noted that cheeses containing adjunct ingredients had lower proportions of *Lactococcus* species. It is thus apparent that high-throughput sequencing-based investigations can provide a valuable insight into the microbial populations of artisanal foods.

1. Introduction

High-throughput sequencing has revolutionised the field of microbial ecology, allowing for a more accurate identification of microbial taxa, including those which are difficult to culture and/or are present in low abundance (Sogin et al., 2006). These technologies have provided a detailed insight into the microbial composition of a wide variety of different ecosystems including sea (Sogin et al., 2006), soil (Roesch et al., 2007) and gut environments (Andersson et al., 2008, Claesson et al., 2009), as well as that of a relatively-small selection of food-associated niches (Dobson et al., 2011, Masoud et al., 2011, Roh et al., 2010). One group of complex microbial environments not assessed, to date, in this way are artisanal cheeses. The complex, fermentation-based nature of cheese means that the microbiota of different cheeses varies considerably. Many of these microbes are also hugely influential with respect to the textural and organoleptic properties of a cheese (Marilley and Casey, 2004). Thus, unsurprisingly there have been considerable efforts made to characterise the microbial populations of cheeses. Traditional culture-independent molecular methods, most frequently the analysis of 16S rRNA genes through denaturing or temporal temperature gradient gel electrophoresis (DGGE/TTGE) (Ercolini, 2004, Ogier et al., 2004), single stranded conformation polymorphisms (SSCP) (Callon et al., 2007) and/or Sanger sequencing (Duthoit et al., 2003), have improved our understanding of cheese microbial population (Quigley et al., 2011). However, we anticipated that the application of high-throughput sequencing could provide an even more detailed understanding of the microbial composition of cheese. Thus we have applied this technology to investigate the microbiota of 62 soft, semi-hard and hard artisanal cheeses, which have been manufactured from unpasteurised or pasteurised cow's, goat's and sheep's milk and of 11 associated naturally developed or smear-ripened rinds.

2. Materials and Methods

2.1 Cheese Collection and Nucleic Acid Extraction

A total of 62 handmade cheeses, 18 soft cheeses, 31 semi-hard cheeses and 13 hard cheeses, manufactured from unpasteurised or pasteurised cows', goat's or sheep's milk, were obtained from artisanal cheese producers and farmer's markets throughout Ireland (Table S1). To facilitate the culture independent analysis of the bacterial composition of these cheeses, their associated rinds, naturally developed or smear-ripened cheese rinds, were also analysed. 1 g of cheese or 1 g of cheese rind (Callon et al., 2006, Coppola et al., 2001, Delbes et al., 2007, Duthoit et al., 2003, Ercolini et al., 2003) was combined with 9 ml 2% tri-sodium citrate and homogenised before DNA was extracted using the PowerFood^{TM} Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) (Quigley et al., 2012).

2.2 PCR amplification of the microbial community 16S rRNA gene

The DNA extracts were used as a template for PCR amplification according to Quigley et al (Quigley et al., 2012). Here, universal 16S primers targeting the V4 region (239 nt long) predicted to bind to 94.6% of all 16S genes were incoporated i.e. the forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The primers incorporated a proprietary 19-mer sequence (GCCTGCCAGCCCGCTCAG) at the 5' end to allow emulsion-based clonal amplification for the 454-pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adapatmer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. The PCR reaction contained 25 µl GoTaq Green Master Mix (Promega), 1 µl of each primer (10 pmol), 5 µl DNA template and nuclease free dsH_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, UK). The amplification programme consisted of an initial denaturation step at 94°C for 2 min, followed by 40 cycles; denaturation at

94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. Amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, United Kingdom). The quantity of DNA extracted was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen, USA) used in accordance with the manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, USA).

2.3 High-throughput sequencing and bioinformatics analysis

The 16S rRNA V4 amplicons were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) according to Roche 454 protocols. Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (Cole et al., 2009). Sequences not passing the FLX quality controls were discarded, the 454 specific portion of the primer were trimmed, the raw sequences were sorted according to tag sequences and reads with low quality scores (quality scores below 40) and short length (less than 150 bp for the 16S rRNA V4 region) were removed as were reads that did not have exact matches with respect to primer sequence. Statistical analysis, to measure the sequencing diversity included Chao1 richness, Shannon diversity and Good's Coverage, as well as, monitoring sequencing abundance using rarefaction analysis, were performed using the MOTHUR package (Schloss et al., 2009). Principal Co-ordinate Analysis, measuring dissimilarities at phylogenetic distances based on Weighted Unifrac was performed using the QIIME suite of programs (Caporaso et al., 2010). Trimmed fasta sequences were assessed by BLAST analysis (Altschul et al., 1990) against a previously published 16S-specific database (Urich et al., 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarisation (absolute cut-off: BLAST bit-score 86, relative cut-off: 10% of the top hit) (Urich et al., 2008). Statistical significance was determined by

the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) using the Minitab® statistical package.

3. Results

3.1 Sequencing and Bioinformatic Analysis

DNA was extracted from a 1g sample size from 62 cheeses and from the rinds of 11 of the cheeses (Table S1). Following total genomic DNA extraction, amplicons of the V4 16S rRNA gene were generated and a total of 116,238 pyrosequencing reads were obtained through 454 sequencing, corresponding to 32,322, 48,388 and 18,340 reads from soft, semi-hard and hard cheeses, respectively, and 17,188 reads corresponding to cheese rinds. Diversity, richness and coverage estimations were calculated for each data set (Table 1; individual sample diversity are presented in Table S2). The Chao1 estimator of species richness indicates good sample richness throughout. The Shannon diversity index, a measurement of overall diversity, indicates a diverse microbiota, while Good's coverage, an estimator of completeness of sampling, highlights good overall sampling with levels of 89-95%. Rarefaction curve analysis, which assesses species richness from the results of sampling, show all samples approaching parallel with the x-axis, revealing that the overall bacterial diversity is well represented (Fig. 1). Principal Co-ordinate Analysis (PCoA), which clusters the communities according to different parameters, in this case cheese type, animal source of milk or whether the milk was pasteurised or not, was examined according to weighted UniFrac distances (Fig. 2). Regardless of the community parameters, there is no definitive split in the microbiota of the cheese. However, the most extreme outliers generally tend to be cheese rinds from cows milk cheeses. No statistical differences were found in OTU at phylum level, however a number of statistical differences were determined at genus level. The gene sequence information has been prepared in a MiXS-MIMARKS metatable (Yilmaz et al., 2011), shown in Supplementary data (Table S3 - available online only).

3.2 The microbial composition of artisanal cheese as revealed by pyrosequencing

In silico analysis of high-throughput sequence data revealed microorganisms corresponding to five phyla in the soft, semi-hard and hard cheeses (Table 2).

These were representatives of four bacterial phyla i.e. the Firmicutes, Proteobacteria, Bacteroidetes and Acintobacteria. Surprisingly, a fifth phylum detected was the fungal phylum Ascomycota. The latter were detected occasionally throughout the cheese samples at a subdominant level, i.e. 0.37-0.50%, and at genus level corresponded almost exclusively with *Penicillium*. Further examination of the *Penicillium* sequence established that it corresponds to that of the mitochondrial 16S rRNA gene of *Penicillium*. Of the four bacterial phyla, Firmicutes dominated in the three cheese types, corresponding to 96%, 95% and 91% of the reads from the soft, semi-hard and hard cheeses, respectively. Proteobacteria (0.91, 3.31 and 1.79%), Bacteroidetes (0.27, 0.25 and 0.21%) and Actinobacteria (1.22, 0.12 and 4.45%) were detected at varying levels throughout (Table 2).

The bacteria present corresponded to 21 different genera (Fig. 3; Table 2), with *Lactococcus* dominating. At the depth of analysis carried out, a total of eight genera were found to be common in all three cheese types (soft, semi-hard and hard). In addition to *Lactococcus* (89.93, 84.45 and 49.56%), *Lactobacillus* (0.65, 7.30 and 17.8%), *Leuconostoc* (1.79, 0.51 and 1.8%), *Pseudomonas* (0.11, 0.03 and 0.49%), *Psychrobacter* (0.58, 0.02 and 0.53%), *Staphylococcus* (0.06, 0.17 and 0.73%), *Arthrobacter* (0.28, 0.08 and 1.1%) and *Faecalibacterium* (0.02, 0.08 and 0.05%) were identified also. Statistically significant differences was observed in the levels of *Lactococcus* (P=0.031) and *Lactobacillus* (P=0.010), with the level of lactococci increasing and the level of lactobacilli decreasing between soft, semi-hard and hard cheese types (Fig. 3). *Vibrio* were found in soft cheese only (0.02%), *Helcococcus* (0.07%), *Halomonas* (0.25%) and *Streptococcus* (0.04%) were found in the semi-hard cheeses only, while *Enterococcus* (0.1%), *Tetragenococcus* (0.05%) and *Clostridium* (0.06%) were found in hard cheeses only. Three genera were shared between soft and semi-hard cheeses. These were *Pseudoalteromonas* (0.06 and 0.03%), *Pediococcus* (0.03% and 0.27%) and *Bifidobacterium* (0.02% and 0.03%). *Brevibacterium* (0.81% and 2.10%) was the only genus shared between soft and hard cheeses and *Prevotella* (0.15% and 0.34%) was the only genus common to semi-hard and hard cheeses.

Some interesting observations were made regarding the influence of the animal source of milk and pasteurisation on the microbial populations present in the resultant cheeses. It was noted that cow's milk cheese contained 21 different bacterial genera (Table 2) whereas goat's milk contained only 8 different bacterial genera and only two bacterial genera, *Lactococcus* and *Lactobacillus*, were detected in sheep's milk cheese. Also, by comparing the bacterial genera present in artisanal cheeses manufactured from unpasteurised, relative to those made from pasteurised, milk, it was apparent that *Halomonas, Helcococcus, Streptococcus, Enterococcus* and *Tetragenococcus* were detected in raw milk cheeses only and that *Clostridium* and *Vibrio* were detected from pasteurised milk cheeses only. A significant difference was noted in the levels of *Lactococcus* (p=0.025) and *Lactobacillus* (p=0.002) between unpasteurised and pasteurised milk cheeses. Further comparisons provided some interesting findings. Cheeses S2 and S3 were produced with milk from the same herd using similar protocols, but differed in that S3 is a feta style cheese and thus has a higher salt content which may explain the absence of *Leuconostoc* and *Pseudomonas* from this cheeses. Similarly, S7 and H9 are produced in the same farmhouse but differ with respect to the level of maturation with associated differences in the proportions of *Lactobacillus* (0.65%, soft cheese; 17.8%, hard cheese). This is reflective of the aforementioned overall greater number of lactobacilli in hard relative to semi-hard and, in turn, soft cheeses. This pattern is also apparent when SH1 and H3-H6, all from the same producers, are compared. In addition, a specific comparison of H4 and H5 is also interesting as these cheeses differ solely on the basis that H5 contains an adjunct ingredient, fenugreek seeds, the presence of which coincides with a reduction in the proportion of lactococci (from 61 to 2%) and increase in lactobacilli (from 34 to 95%). The inclusion of herbs, spices or seaweed was also found to coincide with reduced proportions of lactococci in SH8 relative to SH10-11 and in SH28 relative to SH26, respectively.

3.3 Revealing the microbial composition of the rind of artisanal cheeses

We again used high-throughput sequencing to analyse the microbiota of 11 of the artisanal cheeses rinds (R1-11) (Table S1; Fig. 3). These included

smear/wash ripened rinds, i.e. R1, R7, R8 and R9, naturally developed rinds, i.e. R2-R6 and R11, and one mould ripened rind, R10. *In silico* analysis of sequence data revealed the presence of 19 different genera (Fig. 3; Table 2). While some of these genera, including *Lactococcus, Leuconostoc and Lactobacillus*, corresponded to those also detected in the cheese core, a selection were identified in cheese rinds only. These included *Corynebacterium* (1.2%), *Facklamia* (0.60%), *Flavobacterium* (0.19%) and *Cronobacter* (0.05%). While lactococci remained the most common genus in cheese rinds, the relative proportions of this genus were significantly lower in the rind than in the core. Generally, smear/wash-ripened rinds had particularly low levels of lactococci (1.9-4.8%), while naturally developed rinds had levels of lactococci of up to 98%. It was also apparent that *Psychrobacter* and *Brevibacterium* represented a considerable proportion, i.e. 0.29-57% and 0.67-54.6%, respectively, or ~10% on average, of the total population. The other genera detected, i.e. *Leuconostoc, Lactobacillus, Pseudomonas, Psychrobacter, Pseudoalteromonas, Brachybacterium, Prevotella, Arthrobacter, Streptococcus, Tetragenococcus* and *Facklamia,* corresponded to between 0.03 and 4.14% of reads (Table 2). *Brevibacterium* and *Brachybacterium* had significant differences in the levels present in the rinds of soft, semi-hard and hard cheeses compared to cheese core, p=0.040 and p=0.014, respectively. *Penicillium* was also detected in the cheese rind and in higher proportions than were detected in the cheese core. Finally, we also detected the presence of *Prevotella*, a genera which has previously not been detected in cheese or cheese rinds and noted that *Vibrio* were only identified in rinds of the smear/wash developed variety (p=0.009).

4. Discussion

Here, pyrosequencing based 16S rRNA profiling has provided a detailed insight into the complex microbiota of artisanal cheeses. Its use effectively revealed the presence of a number of taxa not previously associated with specific cheese types or, indeed, of any cheeses. Among those identified for the first time were the genera *Prevotella* and *Faecalibacterium*. *Prevotella* are Gram-negative bacteria from the phylum Bacteroidetes that thrive in anaerobic environments. They are commensals of the rumen and hind gut in cattle and sheep but can also be the cause of periodontal disease as well as other human infections. Members of the genus *Faecalibacterium* are strict anaerobes and have been shown to produce butyrate, D-lactate and formate, as well as utilise acetate (Duncan et al., 2002). While butyrate can contribute positively to cheese development, in high levels this product can induce the late-blowing defect in cheese (Cocolin et al., 2004). D-lactate and acetate are also produced during the development of cheese (Fox, 1999). Further investigations will be required to determine if, at the levels present in cheese, these microbes contribute flavour in a significant way. A third genus which is typically associated with anaerobic gastrointestinal environments, i.e. *Helcococcus*, was also detected but only one cheese, a semi-hard cheese made from unpasteurised cow's milk. *Helcococcus* have been associated with clinical problems in humans (Collins et al., 2004), in cows (Kutzer et al., 2008), sheep (Zhang et al., 2009) and horses (Rothschild et al., 2004) and thus, in this instance, may reflect the sourcing of contaminated milk from an infected animal. Given that, in this study, these insights were gained through the analysis of merely a 1 g sample per cheese, it may be that further investigations of even larger sample sizes and at a greater depth of sequencing will uncover additional genera not previously associated with cheese. Nevertheless, the detection of these anaerobes reveals that the microbiota of cheese is more diverse than previously appreciated, thus further highlighting the benefits of high-throughput sequencing investigations.

We also detected a number of genera not previously associated with specific cheese types. Here we noted that *Arthrobacter* and *Brachybacterium* were detected for the first time in goat's cheese, these are commonly detected on

cheese surface but not, to our knowledge, in the core of goat's cheese. The presence of *Pseudoalteromonas* in soft and semi-hard cow's milk cheeses, as well as cheese rinds was also unexpected. *Pseudoalteromonas* species are usually regarded as marine bacteria (Bozal et al., 2003) and have been detected on the surface of smear-ripened cheese on only one previous occasion (Feurer et al., 2004a). This is the first instance upon which this genus has been detected in a cheese core.

In addition to identifying taxa not previously associated with cheeses or specific cheese types, there were a number of other interesting observations. It was noted that milk source impacted on the number of genera detected, i.e. 21 genera from cow's milk cheese, 8 from goat's milk cheese and 2 from sheep's milk cheese, although this observation may be influenced by differences in the number of samples within each group. Notably, a number of studies have previously established that milk source can influence the type and number of microbes present in a cheese (Coppola et al., 2001, Randazzo et al., 2006). Previous studies have also highlighted the dramatic impact of milk pasteurisation on the microbiota of resultant cheeses (Bonetta et al., 2008, Coppola et al., 2001, Duthoit et al., 2005). Here, through the use of high-throughput sequencing, we also observed differences in cheeses produced from raw and pasteurised milk (Table 2) in that, for example, significant differences in levels of *Lactococcus* and *Lactobacillus* were apparent when these cheese types were compared. For further studies to investigate these differences, it would be interesting to focus on RNA (and thus cDNA), or to employ stains that inactivate DNA from cells which have been killed by the temperature treatment to determine if such approaches provide different results.

Several previous studies have employed other technologies to investigate the impact of salt (Fox et al., 2004), ripening (Martin-Platero et al., 2009) and additional ingredients (Ankri and Mirelman, 1999, Dash et al., 2011, Tajkarimi et al., 2010) on the microbiota of cheese. Here we noted that neither *Leuconostoc* nor *Pseudomonas* were detected in cheeses with a high salt content, significantly increased *Lactobacillus* populations were detected in cheeses from the same farmhouse but which had been ripened to varying

degrees and observed that the inclusion of adjunct ingredients such as herbs, spices or seaweed did impact on microbial composition.

As a consequence of its exposure to the external environment and, in some cases, steps taken during the manufacturing process, the microbiota of the rind of cheese will frequently differ dramatically from that of the rest of the cheese (Feurer et al., 2004b). This presumably reflects the exposure of the cheese rinds to the environment. Many of the bacterial genera detected are commonly identified in cheese rinds and, indeed, *Corynebacterium, Arthrobacter, Brevibacterium* and *Halomonas* have previously been identified on the surface of Irish artisanal cheeses (Mounier et al., 2005). The high proportions of *Psychrobacter* and *Brevibacterium* in the cheese rinds studied here was particularly notable. *Brevibacterium* is known to be involved in the development of cheese rind flavours and smear rind colour (Krubasik and Sandmann, 2000). Although *Psychrobacter* is frequently detected on cheese surfaces, its specific role is unclear. It may contribute to flavour given the ability of strains from this genus to produce branched-chain aldehydes, alcohols and esters (Deetae et al., 2007). The impact of the presence of such high proportions of these bacteria on the cheese surface will require further investigation. The previously unreported or rare phenomenon of *Prevotella*, *Facklamia* (Roth et al., 2011) or *Vibrio* being detected on cheese rinds further highlights the benefits of employing high-throughput sequencing to investigate these populations.

5. Conclusion

Thus, in conclusion, we have employed high-throughput sequencing to investigate the microbiota of 62 Irish artisanal cheeses in greater depth than ever before. We have highlighted for the first time the presence of a number of genera previously undetected in cheese, as well as, detecting genera not typically associated with specific cheese types. These analyses also provide an insight into the influence of different factors on the composition of the artisanal cheese microbiota which can now be investigated in greater depth through the study of cheeses prepared in the laboratory.

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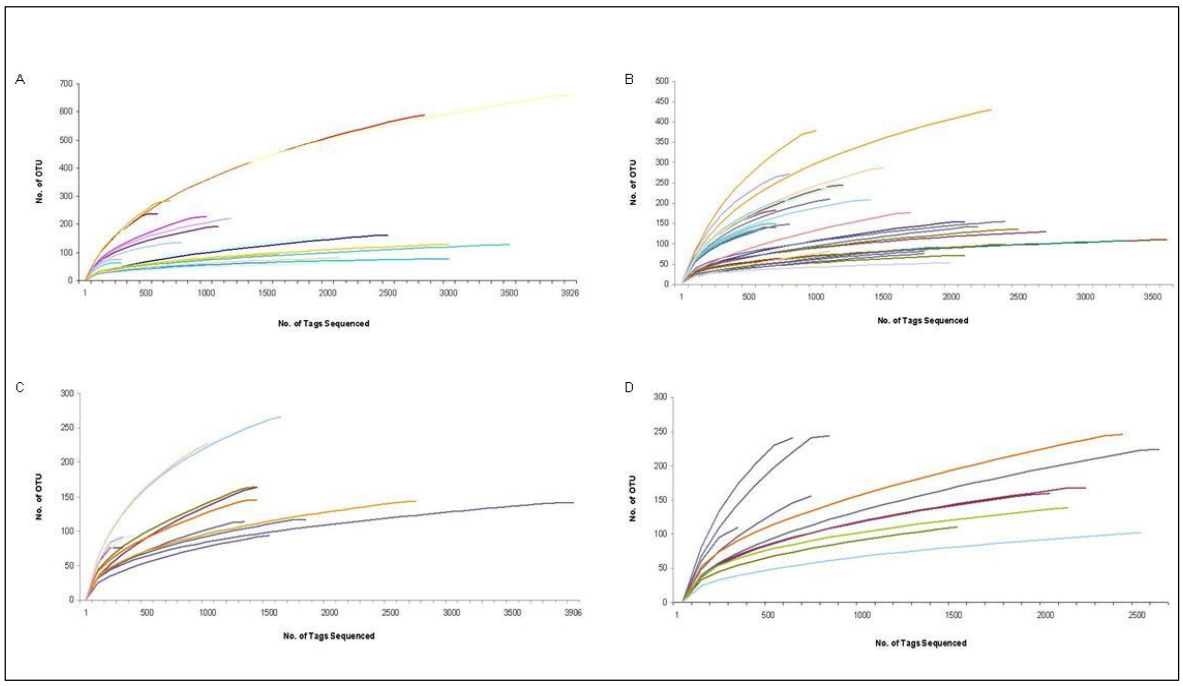

Figure 6: Rarefaction curve of microbial populations from artisanal cheeses and cheese rinds.

Each line represents a cheese sampled and sequenced, A-soft cheeses; B-semi-hard cheeses, C-hard cheeses, D-cheese rinds. The curvature of the line towards the right (or x-axis) shows that a reasonable number of sequenced have been taken, thus sampling is sufficient.

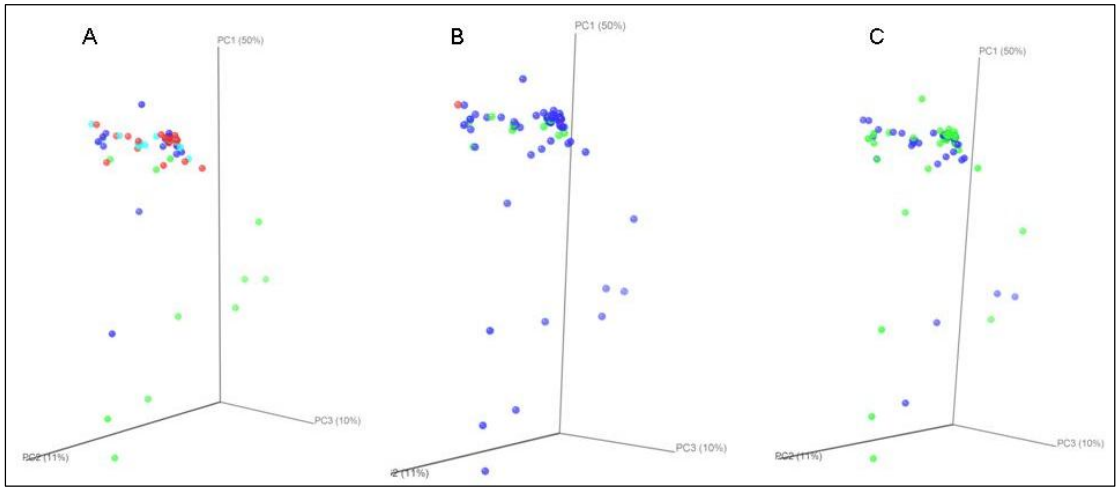

Figure 7: Principal Coordinate Analysis graphs for Weighted UniFrac analysis.

Samples were assessed for different community parameters: A - Cheese Type (soft=light blue, semi-hard=red, hard=dark blue, rind=green); B - Animal Source (cow=dark blue, goat=green, sheep=red); C – Milk (unpasteurised=green, pasteurised=blue).

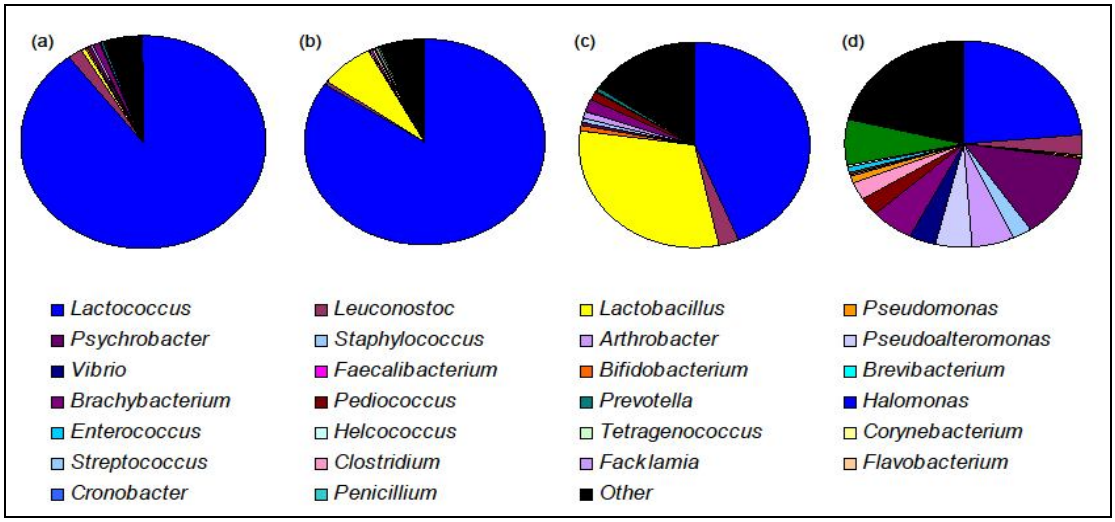

Figure 3: Assignment of cheese microbiota at genus level, according to MEGAN, a= soft cheese; b = semi-hard cheese; c = hard cheese; d = cheese rinds.

Table 1: Average statistical analysis of artisanal cheese, at two different similarity levels, determining sequencing richness, diversity and coverage as analysed by MOTHUR software. The analyses are separated on the basis of cheese type.

	Cheese Type		
Data Set	*Soft*	*Semi-Hard*	*Hard*
Similarity	97%	97%	97%
Chao1 richness estimation	295	315	254
Shannon index for diversity	3.8	4.3	3.7
Good's coverage	92%	90%	91%

Table 2: Percentage of reads calculated from the total phylum reads for each variable assessed.

	Cheese Type				Animal Source			Milk	
	Soft	**Semi-Hard**	**Hard**	**Rind**	**Cow**	**Goat**	**Sheep**	**Unpasteurised**	**Pasteurised**
PHYLUM									
Proteobacteria	0.91	3.31	1.79	26.99	95.5	89.6	98.6	96.1	93.1
Firmicutes	96.03	95.49	91.41	32.14	0.69	1.4	0	0.74	0.83
Acintobacteria	1.22	0.12	4.45	26.02	1.4	6.8	0	1.9	2.5
Bacteroidetes	0.27	0.25	0.21	5.47	0.19	1.1	0	0.22	0.48
Ascomycota	0.49	0.37	0.50	7.13	0.50	0.89	1.38	0.61	0.60
GENUS									
Lactococcus	89.83	84.45	49.56	25.80	77.2	76.0	98.5	72.8	84.4
Leuconostoc	1.79	0.51	1.80	2.57	1.0	2.2	0	1.2	1.1
Lactobacillus	0.65	7.30	17.80	0.20	8.1	1.0	0.08	11.3	0.82
Pseudomonas	0.11	0.03	0.49	0.14	0.07	0.64	0	0.09	0.25
Psychrobacter	0.58	0.02	0.53	9.92	0.28	0	0	0.21	0.23
Staphylococcus	0.06	0.17	0.73	1.98	0.31	0	0	0.30	0.17
Arthrobacter	0.28	0.08	1.10	4.14	0.39	0.85	0	0.49	0.39
Pseudoalteromonas	0.06	0.03	0	3.80	0.03	0	0	0.03	0.02
Vibrio	0.02	0	0	2.84	0.004	0	0	0	0.008
Faecalibacterium	0.02	0.08	0.05	0	0.07	0	0	0.07	0.04
Bifidobacterium	0.02	0.03	0	0	0.02	0	0	0.02	0.009
Brevibacterium	0.02	0	2.10	9.22	0.57	5.4	0	0.82	1.9
Brachybacterium	0.81	0	1.45	3.56	0.39	0.50	0	0.57	0.17
Pediococcus	0.03	0.27	0	0	0.12	0	0	0.17	0.01
Prevotella	0	0.15	0.34	0.03	0.07	0.048	0	0.10	0.16
Halomonas	0	0.25	0	2.46	0.12	0	0	0.1	0
Enterococcus	0	0	0.10	0	0.02	0	0	0.03	0
Helcococcus	0	0.07	0	0	0.03	0	0	0.05	0
Tetragenococcus	0	0	0.05	0.18	0.01	0	0	0.02	0
Corynebacterium	0	0	0	1.20	0	0	0	0	0
Streptococcus	0	0.04	0	0.24	0.01	0	0	0.02	0
Clostridium	0	0	0.06	0	0.01	0	0	0	0.02
Facklamia	0	0	0	0.60	0	0	0	0	0
Flavobacterium	0	0	0	0.19	0	0	0	0	0
Cronobacter	0	0	0	0.05	0	0	0	0	0
Penicillium	0.49	0.37	0.51	12.96	0.50	0.89	1.38	0.61	0.60
Other	5.16	6.14	23.34	17.91	10.67	12.47	0.04	11.1	9.7

Supplementary Data

Table S1: Classification of cheese and rinds sampled.

Soft Cheese				Semi-Hard Cheese				Hard Cheese				Cheese Rind		
Cheese Type	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**
S1[a]	C	UP	-	SH1	C	UP	-	H1	C	UP	-	R1[a]	C	UP
S2	G	UP	-	SH2	C	UP	-	H2	C	UP	-	R2[b]	C	UP
S3	G	UP	-	SH3	C	UP	Oak Smoked	H3[b]	C	UP	-	R3[c]	C	UP
S4	C	UP	-	SH4	C	UP	Cumin Seed	H4	C	UP	-	R4[d]	C	UP
S5	C	UP	-	SH5	C	UP	Fenugreek	H5	C	UP	Fenugreek	R5[e]	C	UP
S6	G	P	-	SH6	C	UP	Black Pepper	H6[c]	C	UP	-	R6[f]	G	UP
S7	G	P	-	SH7	C	UP	Nettle & Garlic	H7	C	UP	-	R7[g]	C	P
S8	C	P	-	SH8	C	UP	-	H8[d]	C	UP	-	R8[h]	C	P
S9[h]	C	P	-	SH9	C	UP	Peppers	H9[f]	G	UP	-	R9[i]	G	UP
S10[j]	C	P	-	SH10	C	UP	Garlic & Herb	H10	C	P	-	R10[j]	C	P
S11	G	P	-	SH11	C	UP	Cumin seed	H11	C	P	-	R11[k]	S	P
S12	G	P	-	SH12	C	UP	Oak Smoked	H12	G	P	-			
S13	G	P	-	SH13	C	UP	Garlic & Herb	H13	G	P	-			
S14	C	P	-	SH14	C	UP	Pepper & Chive							
S15	C	P	Garlic & Herbs	SH15	C	UP	-							
S16	C	P	Oak Smoked	SH16	C	UP	Seaweed							
S17[g]	C	P	-	SH17	C	UP	Garlic							

Table S1 continued:

Soft Cheese				Semi-Hard Cheese				Hard Cheese				Cheese Rind		
Cheese Type	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**	**Adjunct Ingredient**	**Cheese Type**	**Animal Source**	**Milk**
S18[l]	C	P	-	SH18	C	UP	Nettle & Onion							
				SH19	C	UP	Black Pepper							
				SH20[e]	C	UP	-							
				SH21	C	P	-							
				SH22	C	P	-							
				SH23	C	P	-							
				SH24	C	P	Cumin Seed							
				SH25	C	P	-							
				SH26	C	P	-							
				SH27	C	P	Basil & Garlic							
				SH28	C	P	Seaweed							
				SH29[k]	S	P	-							
				SH30	S	P	-							
				SH31	S	P	-							

Classification according to cheese type is based on cheese texture S=soft, SH=semi-hard, H=hard, and the cheese rind R=rind. Other factors considered are: animal source, according to the type of animal that milk used for cheese manufacture was sourced from C=cow, G=goat or S=sheep; and the milk treatment i.e., UP=unpasteurised or P=pasteurised. The subscript letters a-k indicates the cheese and corresponding rind.

Chapter III

The microbial content of raw and pasteurised cow's milk as determined by molecular approaches

The work presented in this chapter was in collaboration with Robert McCarthy. All experimental work, result interpretation and manuscript preparation was carried out in an equal capacity.

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Abstract

The microbial composition of raw and pasteurised milk is assessed by industry on a daily basis. However, many such tests are culture-dependent and thus bacteria that are present at sub-dominant levels and/or that cannot be easily grown in the laboratory may be overlooked. To address this potential bias, we have employed a number of culture-independent techniques, including flow cytometry, real-time qPCR and high-throughput sequencing, to assess the microbial population of milk from a selection of commercial milk producers, pre- and post-pasteurisation. The combination of techniques employed reveals the presence of a previously unrecognised and diverse bacterial population in unpasteurised cow's milk. Most notably, the use of high-throughput sequencing resulted in a number of bacterial genera being identified in milk samples for the first time. These included *Bacteroides, Faecalibacterium, Prevotella* and *Catenibacterium*. Our culture-independent analyses also indicate that the bacterial population of pasteurised milk is more diverse than previously appreciated and that non-thermoduric bacteria within these populations are likely to be in a damaged, non-culturable form. It is thus apparent that the application of state-of-the-art approaches can provide a detailed insight into the bacterial composition of milk and could potentially be employed in the future to investigate the factors that influence the composition of these populations.

1. Introduction

Milk harbours a complex microbial community, including microorganisms of industrial importance, that possess health promoting features or which are of concern from a food quality or safety perspective. Thus, the milk microbiota is the focus of constant attention. Such testing occurs daily on both raw and pasteurised milk and is governed by a variety of methods and standards across different jurisdictions. The microbial composition of milk is influenced by a number of different parameters such as, in the case of raw milk, the microorganisms present in the teat canal, on the surface of teat skin, the surrounding air, feed, as well as other environmental factors including housing conditions, the quality of the water supply and equipment hygiene (Verdier-Metz et al., 2012, Verdier-Metz et al., 2009, Vacheyrou et al., 2011, Braem et al., 2011). The microbiota of pasteurised milk is thought to be determined by the percentage of thermoduric bacteria that survive pasteurisation temperatures and by the bacteria associated with post-pasteurisation contamination, which include psychrotropic bacteria such as *Pseudomonas* (Fromm and Boor, 2004, Ternstrom et al., 1993). The techniques used to identify and count the bacterial populations present generally involve culturing on agar media and are labour intensive and time consuming. Furthermore, microorganisms that cannot be easily cultured in the laboratory, or are present as subdominant populations are not detected using these approaches (Paszyn´ska-Wesołowska and Bartoszcze, 2009). Indeed, comparative culture-based and culture-independent (flow cytometry) analysis of identical milk samples have provided significantly different results (Gunasekera et al., 2002b). Other culture-independent techniques, and in particular those which

are DNA-based, provide a means of examining the bacterial composition of milk without introducing culture-based biases. These DNA-based approaches have included denaturing gradient gel electrophoresis (DGGE) and single stranded conformation polymorphisms (SSCP). These allow a comparison of the relative diversity of different bacterial populations and can to some extent, reveal the identity of specific components (Callon et al., 2007, He et al., 2009). Methods such as quantitative real-time PCR (qPCR) have increasingly been employed, which permit rapid identification and quantification, albeit only of specific target microbes (Rodríguez-Lázaro et al., 2005, He et al., 2009). Even more recently, there have been significant developments in the field of microbial ecology as a consequence of the development of culture-independent analysis *via* high-throughput DNA sequencing. This approach can provide a more in-depth insight into the diversity and dynamics of entire microbial communities (Andersson et al., 2008, Delmont et al., 2012, Quigley et al., 2011, Sogin et al., 2006). In a few exceptional cases this technology has been applied to dairy based environments, such as cheese (Alegria et al., 2012, Masoud et al., 2011, Quigley et al., 2012b) and kefir (Dobson et al., 2011).

The aim of this study was to provide a detailed insight into the microbial composition of raw and pasteurised milk, sourced from a variety of facilities using high-throughput DNA sequencing, in combination with other culture-independent approaches.

2. Materials and Methods

2.1 Strains and culture conditions

The strains used in this study were *Lactococcus lactis* HP and *Pseudomonas aeruginosa* PAO-1 (Teagasc Food Research Centre Culture Collection). *L. lactis* was grown in M17 broth with 0.5% glucose (GM17) at 30°C and *P. aeruginosa* was grown in LB broth and on 1% LB agar plates at 37°C.

2.2 Flow cytometry

The viability of microbial populations found in raw and pasteurised milk samples obtained from the Moorepark dairy heard were investigated using flow cytometry (FCM). FCM analyses were performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, USA) utilising two air-cooled lasers, a 20-mW solid state (emission, 488 nm) and a 17-mW HeNe (emission, 633 nm), and five sensors for the detection of forward (FSC) and sideward (SSC) light scatter, green (FL1, 525 nm), yellow (FL2, 575 nm), and far red (FL3, 695 nm) fluorescence. FCM detector and threshold settings were established using a series of control studies. Here, raw milk (from the Moorepark dairy herd) spiked with *L. lactis* were subjected to heat treatments at 80°C for 30 min and 3 h. Prior to analysis, proteins and lipids were removed from milk samples using a modified version of the procedure described by (Gunasekera et al., 2000). Briefly, milk was treated with 20 AU ml^{-1} proteinase K, and 500 µl of 0.05% Triton X-100 at 37°C with shaking for 45 min. Samples were centrifuged at 18,000 *g* for 10 min, following which the milk fat layer and supernatant were removed. The resulting pellet was washed twice and resuspended in 1 ml filtered (0.22µm) phosphate buffered saline (PBS)

(Invitrogen, Life Technologies, Carlsbad, California). Viability testing was performed using BD Cell Viability Kit (BD Biosciences). Samples were stained with Propidium Iodide (PI; 41nM) for 10 min on ice, followed by Thiazole Orange staining (TO; 8.5µM) for 15 min in the dark. Cell samples were delivered at the "low" flow rate, corresponding to 500 to 1000 cells/s, until 10,000 cells were measured. Fluorescence signals were recorded by using the following detector settings: FSC, 300; SSC, 300; FITC, 600; and PI, 500. A threshold was set at a SSC signal of 300 to reduce background noise deriving from cellular debris and traces of milk components remaining following treatment. Following the generation of threshold parameters, commercial raw and pasteurised milk samples were assayed to assess the relative proportion of live:dead microbes. Data analysis was performed using the FACSDiva software v.5.0.2 (BD Biosciences).

2.3 Collection and treatment of milk samples

Cow's milk, both pre- (i.e. raw) and post-pasteurisation, were obtained from six industrial facilities around Ireland, three samples of each milk type were collected for analysis. Fresh unpasteurised milk was also obtained from the dairy herd at Teagasc Research Centre, Moorepark and was pasteurised in-house using a Microthermics Heat Exchanger (Microthermics Wellington Ct. USA.) at 72°C for 15 sec followed by rapid cooling to 4°C. All milk samples were transported to the laboratory on ice before storage at -20°C. Milk samples were defrosted at 4°C prior to use.

2.4 High-throughput sequencing and bioinformatics analysis

Prior to extraction of DNA, milk (both raw and pasteurised) were treated with 100 µg ml^{-1} of nucleic acid stain ethidium monoazide (EMA) (VWR, Dublin 15, Ireland) (Rudi et al., 2005) to inactivate DNA not associated with living microbes (supplementary data). Total DNA was then isolated from 1ml of each raw and pasteurised milk sample using the PowerFood Microbial DNA extraction kit (MO BIO Laboratories, Carlsbad, CA) in accordance with manufacturer's instructions. Additionally, a 10 min incubation step at 70°C was incorporated to improve DNA yield, as described previously (Quigley et al., 2012a). The DNA extracts were used as a template for PCR amplification of 16S rRNA tags (V4 region; 239 nt long) using universal 16S rRNA-targeting primers predicted to bind to 94.6% of all 16S rRNA genes, forward primer F1 (5'-AYTGGGYDTAAAGNG) and a combination of four reverse primers R1 (5'-TACCRGGGTHTCTAATCC), R2 (5'-TACCAGAGTATCTAATTC), R3 (5'-CTACDSRGGTMTCTAATC) and R4 (5'-TACNVGGGTATCTAATC) (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp). The PCR reaction contained 25 µl GoTaq Green Master Mix (Promega Corporation, Madison, USA), 1 µl of each primer (10pmol), 5 µl DNA template and nuclease free dsH_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, UK). Following a hot start of 10 min at 95°C to denature DNA, the amplification programme consisted of an initial step at 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also included. PCR amplicons were visualised on a 1.5% agarose

gel. Amplicons were cleaned using the AMPure XP purification system (Beckman Coulter, Takeley, United Kingdom). The quantity of DNA extracted was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen, USA) according to manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, USA). The ND3300 excites in the presence of dsDNA bound with Picogreen® at 470nm and monitors emission at 525nm.

The 16S rRNA V4 amplicons were sequenced on the Teagasc454Sequencing Platform (Genome Sequencer FLX; Roche Diagnostics Ltd, West Sussex, UK). Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (Cole et al., 2009). Sequences not passing the FLX quality controls were discarded, the 454 specific portion of the primer were trimmed, the raw sequences were sorted according to tag sequences and reads with low quality scores (quality scores below 25) and short length (less than 150 bp for the 16S rRNA V4 region) were removed as well as reads that did not have exact matches with the primer sequence. Statistical analysis, to measure the sequence diversity included Chao1 richness, Shannon diversity and rarefaction for monitoring sequencing abundance, were performed using the MOTHUR package (Schloss et al., 2009). Principal Co-ordinate Analysis, measuring dissimilarities at phylogenetic distances based on Weighted Unifrac was performed using the QIIME suite of programs (Caporaso et al., 2010). Trimmed fasta sequences were BLAST (Altschul et al., 1990) against a previously published 16S-specific database (Urich et al., 2008) using default parameters. The resulting BLAST output was parsed using MEGAN (Huson et

al., 2007). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarisation (absolute cut-off: BLAST bit-score 86, relative cut-off: 10% of the top hit) (Urich et al., 2008). Statistical significance was determined by the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) using Minitab® statistical package.

2.5 Real-time quantitative PCR-based analysis

To facilitate the absolute quantification of *Pseudomonas* in milk samples, a plasmid standard was first created. Here, a PCR product from within the 16S rRNA gene of *P. aeruginosa* was generated using 16S rRNA targeting genus-specific primers i.e. forward 5'- GACGGGTGAGTAATGCCTA- 3' and reverse 5'- CACTGGTGTTCCTTCCTATA- 3', according to (Spilker et al., 2004). Purified amplicons were cloned into the pCR®2.1-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad, California) in accordance with manufacturer's instructions. Following cloning, the complete vector was transformed into chemically competent TOP-10 *E. coli* cells (Invitrogen) and harvested on LB media containing 50 µg ml^{-1} ampicillin. The accuracy of the cloned amplicon was confirmed by restriction analysis and DNA sequencing. Quantitative real-time PCR (qPCR) standards were prepared following the linearization of plasmid DNA with *Kpn*I restriction enzyme and quantification with the Nanodrop ND-1000 (Thermo Fisher Scientific Inc). A standard curve was then generated via a series of dilutions

from 10^9 to 10^2 copies μl^{-1} DNA. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for quantification according to the manufacturer's instructions. Each PCR reaction contained 10 µl SYBR green master mix, 1 µl of both forward and reverse primer (10pmol), 1 µl of DNA and was made up to a final volume of 20 µl with nuclease free dsH_2O. The PCR conditions were as follows: an initial denaturation at 95°C for 10min, followed by 35 cycles of denaturation at 95°C for 10 sec, annealing at 54°C for 5 sec and elongation 72°C for 25 sec. qPCR assays on test samples and controls were performed in triplicate. To facilitate quantification by qPCR, it was necessary to first generate a standard curve relating cfu to copy number of 16S rRNA genes. Based on the amplification of DNA, which was isolated from *Pseudomonas* cultures of known cfu/ml ($3x10^{7,}$ $3x10^6$, $3x10^5$ cfu ml^{-1}), against a standard curve generated from the Lightcycler 480 (copy number μl^{-1}), a formula was generated to correct copy number values for cfu ml^{-1} i.e.

$$\frac{(C/\mu l)(TV)}{TCN} \times \frac{T\ cfu/ml}{1} = cfu/ml(S)$$

Where; C/µl = Copy number μl^{-1}, TV = Template volume, TCN = Total copy number of the standard used, T cfu/ml = Total cfu ml^{-1} of standard used and cfu/ml(S) = cfu ml^{-1} of test sample. For example, results generated from comparing the "copy number" standard curve to DNA extracted from cultures of known cfu ml^{-1} showed that an average value of, for example, 4.0×10^9 amplicon copies corresponded to 3.2×10^8 cfu ml^{-1} of *P. aeruginosa* and so in the calculation above "TCN" = $4.0x10^9$ and "T cfu/ml" = $3 x10^8$.

3. Results

3.1 Flow cytometry highlights the proportion of viable microorganisms in pasteurised milk

Flow cytometry was employed to compare the relative proportion of viable and dead cells in milk (both raw and pasteurised). Using this approach, the reduction in FITC fluorescence intensity when compared to control samples was deemed indicative of injury or death. The relative dot plot location of live and dead cells was first determined by spiking milk with what is regarded to be a thermosensitive species, i.e. *L. lactis* (Dumalisile et al., 2005), at $1x10^6$ cfu ml^{-1} (Figure 1A), as a control. As raw milk was used for these control studies, the innate microbiota of the milk remained intact. However, the high inoculum of *Lactococcus* ensured that the majority of events detected (> 90%) would correspond to *L. lactis* cells. Following the identification of viable cell populations (Figure 1A), positions occupied of injured/dead populations were determined following the extreme heat treatment of the *L. lactis* spiked milk at 80°C for 30 min (Figure 1B). This extensive heat treatment resulted in the inactivation of the vast majority of cells present (99.52% of events), with just a small number (0.48% of events) remaining in the “live” gated area. Having established the relative locations of viable and dead cells on this control plot, the focus switched to a direct comparison of raw and pasteurised milk. Raw milk was shown to contain mostly viable cells but some dead/injured cells were also detected (Figure 1C). The cell populations present in the pasteurised milk sample were found to be predominantly non-viable, 10.7% were contained in the viable gate (Figure 1D). As expected, statistical analysis of these results revealed significant reductions in viable cell populations in

pasteurised milk ($p<0.01$) compared with raw milk samples. Nonetheless, it is evident that a viable, microbial population was present in the pasteurised milk samples. To characterise this population a high-throughput DNA sequencing strategy was initiated.

3.2 High-throughput sequencing reveals the presence of taxa not traditionally regarded as components of the milk microbiota

Raw and pasteurised cow's milk was obtained from seven facilities located throughout Ireland. In each case the same milk, pre- and post-pasteurisation, was compared. To overcome the bias associated with the ability of PCR-based methods to amplify all DNA present, including DNA from dead cells, we incorporated the use of EMA. Following control tests of EMA (supplementary data) we determined that the use of this stain is critical to ensure that DNA from dead cells does not lead to false positives. Following DNA extraction from these milk samples, amplicons of the V4 region of the 16S rRNA gene were generated and sequence reads were obtained by high-throughput sequencing for phylogenetic assignment. A total of 48,837 pyrosequencing reads were obtained by 454 sequencing. Diversity and richness estimations were calculated for each data set (Figure 2). The Chao1 estimator of species richness indicates good sample richness throughout. The Shannon diversity index, a measurement of overall diversity, indicates a diverse microbiota (Figure 2A). Rarefaction curve analysis revealed that the overall depth of sequencing is sufficient (Figure 2B). Kruskal-Wallis-based analysis of sequencing diversity values revealed that the values for raw and pasteurised milk samples are not significantly different. This highlights that while

pasteurisation is changing the number of bacteria present, the taxa present in the pasteurised milk are similar to those in the raw milk sample. This is also indicated by PCoA analysis, employing Weighted Unifrac distance matrix at OTU, which revealed no definitive split between raw and pasteurised milk samples (data not shown). When examined in combination, sequence reads corresponding to four distinct bacterial phyla, i.e. Proteobacteria, Bacteroidetes, Firmicutes and Actinobacteria, were detected in raw milk while five bacterial phyla, i.e. those present in raw milk in addition to Acidobacteria, were detected in pasteurised milk (Figure 3A and 2B). Notably, reads corresponding to the fungal phylum Ascomycota were also detected at low levels in both raw and pasteurised milk. At genus level these fungal sequences corresponded almost exclusively to *Penicillium* sp. Further examination of the *Penicillium* sequences using BLAST established that they correspond to that of the mitochondrial 16S rRNA gene of *Penicillium*. Of the bacterial phyla, Firmicutes was found to dominate in both raw and pasteurised milk (80.51% and 84.04%, respectively). There was a significantly greater proportion of Proteobacteria in raw, relative to pasteurised, milk (9.20% versus 5.75%; p=0.02), and decreases in the proportions of Bacteroidetes and Actinobacteria were also apparent. Further analysis of DNA sequence data (Figure 3C and 3D) highlighted that, at the family level, both raw and pasteurised milk were dominated by *Streptococcaceae* with read abundance being 57% in raw milk and 77.69% in pasteurised milk. As expected at genus level these reads were found to predominantly correspond to *Lactococcus* sp. (Figure 3F) (data corresponding to the individual milk facilities can be observed in supplementary data). A low proportion of *Streptococcus* sp. was

also apparent. Other members of the lactic acid bacteria, i.e. *Leuconostocaceae* and *Lactobacillaceae*, were also detected. The population most dramatically reduced as a consequence of pasteurisation were the *Pseudomonaceae*. These decreased significantly from 26.34% in raw milk to 4.1% in pasteurised milk (p=0.055) (Figure 3E-F). We further selected *Pseudomonas* for quantification by qPCR. The selection of *Pseudomonas* as a representative was as a consequence of its contribution to the spoilage of milk (Ternstrom et al., 1993) and the high-throughput sequencing-based data highlighting it as a species which decreased significantly in overall relative proportions following pasteurisation. Raw milk was found to contain 1.21 $x10^4$ cfu ml^{-1} of *Pseudomonas* on average, whereas the pasteurised samples showed a significant ($p < 0.001$) reduction in viable *Pseudomonas* to $2.07x10^2$ cfu ml^{-1}. This reduction, though significant, is less dramatic than that observed following assessment of the efficacy of pasteurisation on *Pseudomonas* numbers using culture based approaches, which demonstrated that pasteurisation eliminates *Pseudomonas* from milk spiked with 1 x 10^6 cfu ml^{-1} *Pseudomonas aeruginosa* (data not shown). A number of other microbes often associated with dairy environments were also detected at low levels. These included the *Brevibacteriaceae*, *Corynebacteriaceae* and *Staphylococcaceae*, all of which were identified in raw milk only. A number of other families were found to be present in both raw and pasteurised milk. These included the *Bifidobacteriaceae*, *Flavobacteriaceae*, and *Micrococcaceae*, the latter corresponding to *Arthrobacter* at genus level. A number of typically gut associated bacteria were also present in the milk samples. These included the *Enterobacteriaceae*, with reads corresponding to

Cronobacter at the genus level. These were detected at low levels in pasteurised milk only suggesting possible post-pasteurisation contamination. Also present were the *Clostridiaceae* and *Ruminococcaceae*, which, in the latter case, corresponded to the genera *Ruminococcus* and *Faecalibacterium*. Two families detected, i.e. *Pasteurellaceae* and *Neisseriaceae*, are more typically associated with the colonization of mucosal surfaces of many animals, while two families of more typically marine bacteria, i.e. *Pseudoalteromonaceae* and *Vibrionaceae*, were also detected. Finally, a number of other bacterial families that are not regarded as typical members of the milk microbiota were also detected. These include the *Bacteriodaceae*, the *Porphyromonadaceae* (p=0.046) and the *Moraxcellaceae* (p=0.040). The latter corresponded to two genera, i.e. *Acinetobacter* and *Psychrobacter*, with *Acinetobacter* being found in raw milk only.

4. Discussion

Here we applied culture-independent techniques, including flow cytometry, high-throughput DNA sequencing and real-time quantitative PCR to uncover the bacterial profile of cow's milk, pre- and post-pasteurisation. High-throughput DNA sequencing based 16S rRNA profiling revealed that cow's milk, both raw and pasteurised contains a diverse microbial population. The microbial populations present in the milk samples differed according to the facility from which it was sourced (supplementary data) but generally milk samples were dominated by LAB and *Pseudomonas.* Although many of the microbes detected are those previously associated with milk or dairy products, a number of the bacteria isolated have not previously been detected in milk. These include *Bacteroides, Parabacteroides* and *Faecalibacterium,* which are more frequently regarded as gut microbes that thrive in anaerobic environments, as well as *Prevotella* and *Catenibacterium.* The role of *Bacteroides* in gut health is controversial and has been the subject of much debate. It has been suggested that *Bacteroides* may play a significant role in the pathogenesis of allergies during infancy (Kirjavainen et al., 2001, Suzuki et al., 2008), while others have shown recently that it can provide protection against cow's milk allergies in mice trials (Rodriguez et al., 2012). The species *Parabacteroides* represents a reclassification of microbes that were previously regarded as being *Bacteroides* (Sakamoto and Benno, 2006). *Faecalibacterium* are commensal human gut bacteria with apparent anti-inflammatory activity (Sokol et al., 2009, Sokol et al., 2008), which may ultimately be investigated with a view to its use as a probiotic to treat Crohn's disease, colitis and irritable bowel syndrome (Kirjavainen et al.). *Prevotella*

are commensal microbes in rumen and hind gut of cow's and sheep and in humans, where they help the breakdown of proteins and carbohydrates (Yildirim et al., 2010). It is also associated with periodontal disease in humans (Maeda et al., 1998). Although *Catenibacterium* are also associated with the human gut, little is known about this genus (Kageyama and Benno, 2000). The impact of these microbes on milk, milk products or hosts, following the consumption of milk, remains unknown but, on the basis of these results, merits further attention.

Of the microbes which have previously been associated with milk, psychrotolerant microorganisms present a considerable challenge to the dairy industry due to their ability to accelerate spoilage at storage conditions widely used in dairy production. *Pseudomonas* spp. are regarded as the predominant psychrotolerant bacteria in raw milk and a major contributor to milk spoilage. Previously, culture based techniques suggested that these microbes are heat sensitive and usually do not survive pasteurisation (Cousin, 1982, Ranieri et al., 2009). We confirmed that culture based approaches indicate that pasteurisation eliminates *Pseudomonas* from sterile milk spiked with $1x10^6$ cfu ml^{-1} of *Pseudomonas aeruginosa.* However, culture-independent methods reveal an overall reduction in, rather than elimination of, the *Pseudomonas* population following pasteurisation suggesting that "damaged" but non-viable cells are still present. Such cells are potentially metabolically active but are unable to be cultured on solid media. This finding is comparable to those revealed in a previous study (Gunasekera et al., 2002a), which established that low numbers of *Pseudomonas* survive commercial pasteurisation and so may contribute to the *Pseudomonas* population in pasteurised milk. These

and other pyschrotolerant bacteria may be overlooked when assessed by culture based approaches as a consequence of being in a “viable but non culturable” (VBNC) or highly stressed state.

5. Conclusion

In conclusion, this study reveals the existence of a diverse microbial population in cow's milk, including the presence of a number of bacteria not previously observed in milk. Furthermore, changes in microbial diversity following pasteurisation were seen to be less significant than previously expected following assessment of culture based analysis (Fromm and Boor, 2004, Ternstrom et al., 1993). Finally, the data generated in this study suggests the potential for microbes, usually considered to be eliminated by pasteurisation, to survive commercial pasteurisation, this work suggests that these cells are in a highly stressed or VBNC state. While the reductions in numbers which does occur, combined with the stressed state of surviving cells, ensures the safety of pasteurised milk, the apparent presence of these populations in commercial milk, and their potential impact on milk quality, shelf-life and milk-based products is intriguing and merits closer attention.

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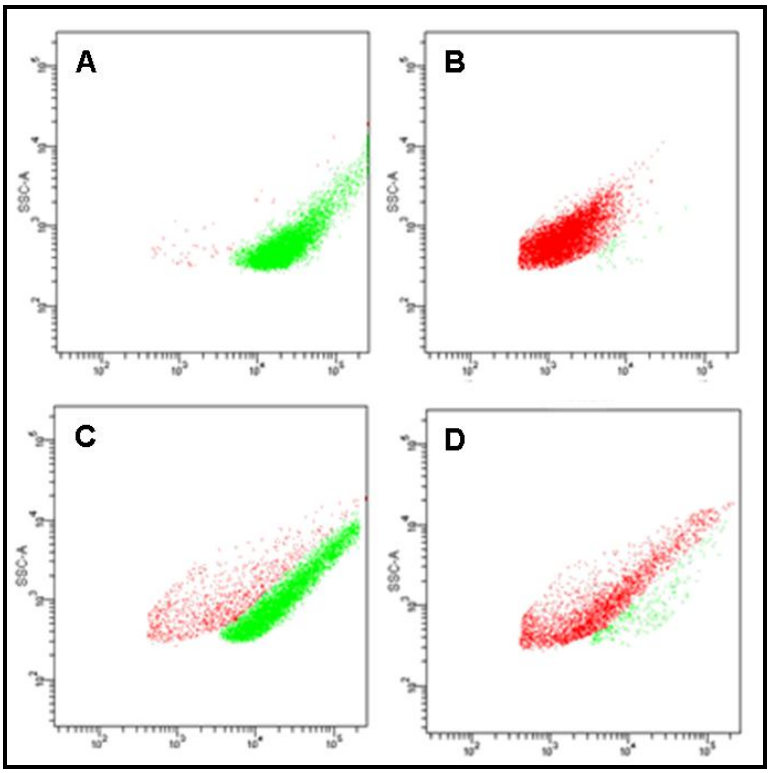

Figure 1: Flow cytometric analysis to determine the relative proportions of live and dead bacteria in milk.

A-B. Treated milk spiked with $1x10^6$ cfu ml^{-1} *Lactococcus lactis* pre (A – viable cell position) and post (B – dead cell position) heat treatment (80°C 30 min). **C.** Commercial raw milk containing mostly viable cells. **D.** Commercially pasteurised milk samples in which a significant reduction in viable cells, compared to commercial raw milk, is apparent. In all cases the data is representative of triplicate data.

A

Data Set	Raw Milk	Pasteurised Milk
Similarity	97%	97%
Chao1 richness estimation	1595	1977
Shannon index for diversity	6.41	6.59

B

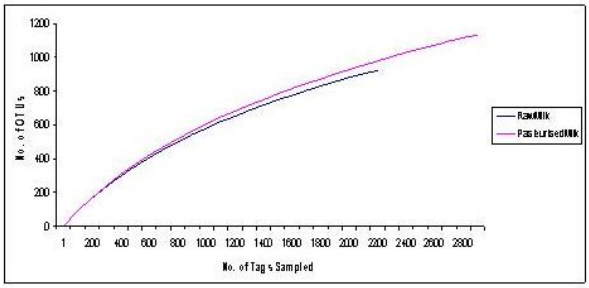

Figure 2: Analysis of the alpha diversity of the milk microbiota.

A. Rarefaction curve analysis of bacterial diversity. **B.** Estimation of diversity of raw and pasteurised milk at 97% similarity level.

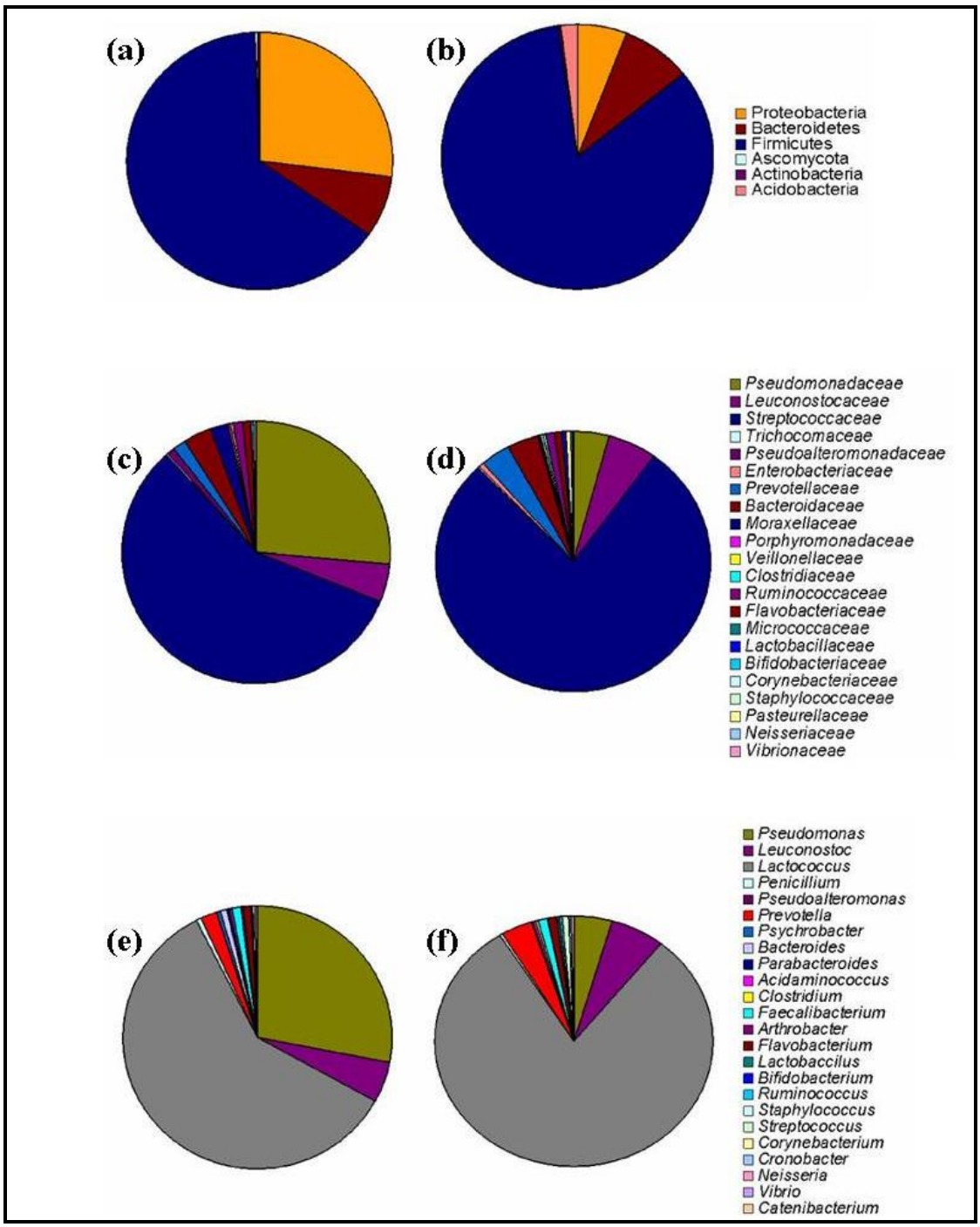

Figure 3: Taxonomic assignment of the milk microbiota as determined by MEGAN.

(a) Raw milk, phylum **(b)** Pasteurised milk, phylum **(c)** Raw milk, family **(d)** Pasteurised milk, family **(e)** Raw milk, genus **(f)** Pasteurised milk, genus.

Supplementary Data

Use of Ethidium Monoazide to differentiate live and dead bacterial population in milk

Prior to DNA extraction milk samples were treated with ethidium monoazide (EMA) to inactivate DNA associated with dead microbes. EMA is a fluorescent stain which intercalates with nucleic acids after photolysis. It penetrates the cell wall of dead cells binding to the DNA. In mixed bacterial population, such as milk, the DNA from viable cells is unstained by EMA. The cells bound with EMA lead to signal reduction in the PCR thus preventing amplification (Rudi et al., 2005). For this, milk was treated with 100 µg ml^{-1} EMA, the sample was incubated in the dark at room temperature for 5 min, activation of EMA occurred by exposing the samples to a 500W halogen light. For this process, samples were placed on ice to reduce overheating of the samples by light, which was located 15cm away. Exposure occurred for 1 min, followed by a 1 min period without light. This process continued until a combined total of 4 min of light exposure was achieved.

To determine the efficiency of EMA in suppressing amplification of dead bacterial DNA a control study was carried out. Here 1 ml of raw milk was spiked with approx $1x10^6$ cells from overnight culture. One ml of the spiked milk was heat treated to mimic pasteurisation at 72°C for 1 min and rapidly cooled on ice. Raw milk and 'pasteurised' milk samples were then treated with 100 µg ml^{-1} EMA, as described above. Samples not treated with EMA, were also subjected to subsequent assessment, for comparative analysis, in triplicate. Total DNA was extracted from all samples using the PowerFood Microbial DNA extraction kit (MO BIO Laboratories, Carlsbad, CA and this DNA was used as a template for PCR amplification of 16S rDNA using the universal primers forward primer F1 (5'-AYTGGGYDTAAAGNG) and reverse primer R1 (5'-TACCRGGGTHTCTAATCC). The PCR reaction contained 25 µl GoTaq Green Master Mix (Promega), 1 µl of each primer (10pmol), 5 µl DNA template and H_2O to give a final reaction volume of 50 µl. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, UK). Following a hot start of 10 min at 95°C to denature DNA, the amplification programme consisted of an initial step at 94°C for 2 min, followed by 40 cycles; denaturation at 94°C for 1 min, annealing at 52°C for 1min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 min was also

included. PCR amplicons were visualised on a 1.5% agarose gel. Notably, the intensity of the resultant 16S rRNA amplified products differed, being marginally and dramatically reduced in the cases of the raw and pasteurised samples, respectively (Figure S2). Thus, a significant proportion, but not all, DNA from pasteurised milk originates from dead cells and thus the treatment of pasteurised milk with EMA prior to amplicon generation and sequencing is critical to ensure that DNA from dead cells does not lead to false positives.

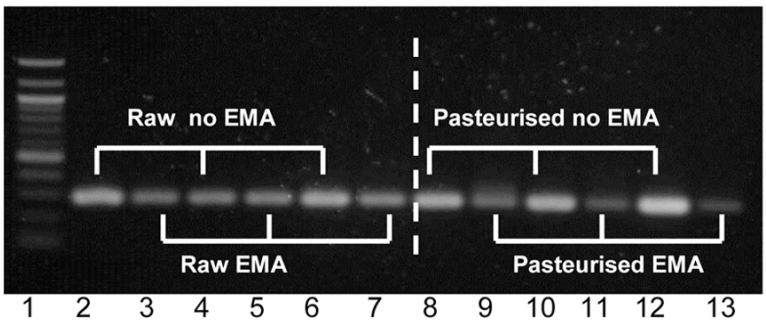

Figure S1: Assessment of the impact of ethidium monoazide (EMA) treatment of template DNA extracted from raw and pasteurised milk on subsequent PCR amplification as revealed by gel electrophoresis imaging.
Lane 1:100bp molecular weight marker.

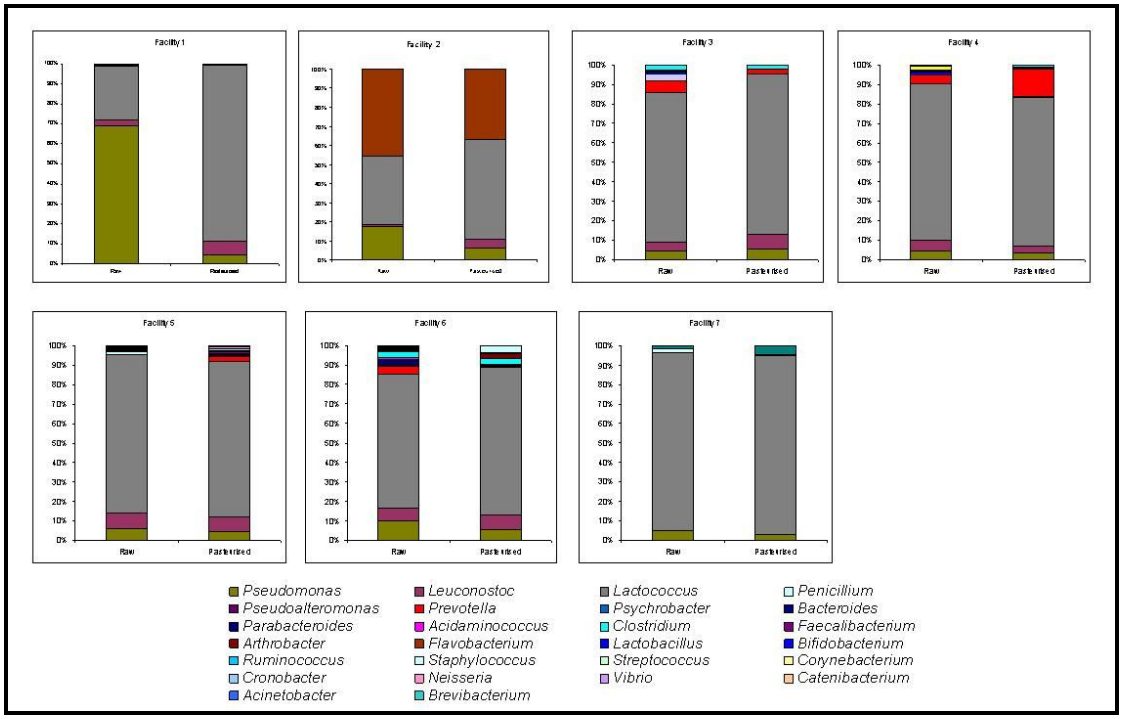

Figure S2: Microbial profile (genus level) for raw and pasteurised milk samples for each of the seven individual facilities tested.

16S rRNA sequencing reveals that each facility has its own unique profile but that the lactococci are consistently dominant. Furthermore although *Pseudomonas* are consistently present, the proportion of the microbial population that they constitute varies and is particularly high in Facility 1 (raw milk).

Chapter IV

Thermus thermophilus and its role in the pinking defect in cheese

Abstract

Pink discolouration in cheese is a global problem. Here we applied high-throughput DNA sequencing to determine if this phenomenon is dictated by the microbial content of cheeses. More specifically, we assessed three types of cheeses in which the problem is encountered, including Swiss-type cheese, “thermophilic”-Cheddar type cheese and Cheddar cheese with coloured annatto. Sequencing data revealed the presence of *Thermus* in Swiss-type and “thermophilic”-Cheddar type cheeses, at a significantly higher level than in control cheeses (p=0.002 and 0.004, respectively). In contrast, the microbial composition of the Cheddar cheese with coloured annatto revealed no differences in the microbial population. The latter observation is consistent with the literature which suggests that the defect in these cheeses is a different phenomenon and is most likely due to physicochemical factors. We developed a rapid qPCR based method to detect and quantify *Thermus*, which was classified at the species level as *Thermus thermophilus*. The levels of this bacterium present in Swiss-type and “thermophilic”-Cheddar type cheeses was 10^3 cfu g^{-1} in defect cheeses. In contrast, it was not present in Swiss-type control cheeses and was at low levels (10^1 cfu g^{-1}) in “thermophilic”-Cheddar type control cheeses. Following the production of three experimental Swiss-type cheeses spiked with *T. thermophilus*, and of control cheeses, it became apparent that the pink discolouration occurred in the spiked cheeses only. This pinking was significantly greater when the levels of the starter cultures were adjusted i.e. an increase in *Lactobacillus helveticus* with or without a decrease in *Streptococcus thermophilus*. Finally, we monitored the dairy processing environment for *T. thermophilus* and identified its presence at multiple locations, with hot water representing the most likely source of contamination. This work has made significant progress in understanding the cause of the pink discolouration in cheese, a problem which has been in existence for many decades.

1. Introduction

The pinking defect in cheese is a global problem (Daly et al., 2012). It impacts on a range of ripened cheeses, including Swiss, Cheddar and Italian-type cheese (Carini et al., 1979, Giuliano et al., 2003, Martley and Michel, 2001, Park et al., 1967, Pelaez and Northolt, 1988, Shannon and Olson, 1969) as well as in ripened cheeses coloured with the food colouring, annatto (Andersen et al., 2006, Hong et al., 1995a, Hong et al., 1995b, Mortensen et al., 2004, Mortensen et al., 2002, Shumaker and Wendorff, 2007). Its appearance can result in the downgrading or rejection of cheese and a consequential economic loss to producers (Daly et al., 2012). Pink discolouration defect can manifest in a number of ways depending on the cheese-type; at the surface of the cheese block, in patches or all over the block surface, sporadically distributed within the cheese block, or as a uniform pink border occurring below the external surfaces of the cheese block conferring a pinked ring appearance (Daly et al., 2012). While the cause of this defect is unknown, the topic has been the subject of much debate. It has been suggested that the pink defects are caused by physicochemical factors (Govindarajan and Morris, 1973, Martley and Michel, 2001, Paramita and Broome, 2008, Shumaker and Wendorff, 2007), though a microbial basis has also been proposed (Betzold, 2004, Shannon and Olson, 1969). Indeed, in the latter case, it has been noted that cheeses containing specific starter cultures, and strains of lactobacilli and propionic acid bacteria (PAB) in particular, are more likely to have a pink discolouration (Bottazzi et al., 2000, Park et al., 1967, Shannon and Olson, 1969).

The recent advances in high-throughput DNA sequencing have transformed the field of microbial ecology. These technologies have provided a detailed insight into the microbial composition of a wide variety of different ecosystems including sea, soil and gut environments (Andersson et al., 2008, Roesch et al., 2007, Roh et al., 2010), as well as a selection of food-associated niches (Dobson et al., 2011, Roh et al., 2010, Sakamoto et al., 2011) including, more recently, dairy-based foods (Alegria et al., 2012, Ercolini et al., 2012, Masoud et al., 2011, Quigley et al., 2013, Quigley et al., 2012b), revealing novel findings in all cases. Here we employ high-throughput

sequencing to reveal and compare the microbial composition of cheeses with a pink defect relative to controls and, ultimately, identify the microbial component responsible for this significant problem.

2. Materials and Methods

2.1 DNA extraction from cheeses

Commercial cheese samples, with (defect cheese) or without (control cheese) pinking discolouration were supplied by four cheese facilities (Table 1). For nucleic acid extraction, 1 g of cheese from the defect or control cheese was combined with 9ml 2% tri-sodium citrate and homogenised before DNA was extracted using the PowerFood™ Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) (Quigley et al., 2012a). As described previously (Quigley et al., 2012a), additional steps, whereby the homogenate was treated with 50 µg ml^{-1} lysozyme and 100 U mutanolysin at 37°C for 1 hour followed with protein digestion by adding 250 µg ml^{-1} proteinase K and incubating at 55°C for 1 hour, were added to the standard manufacturer's instructions.

2.2 Generation of 16S rRNA amplicons for high throughput sequencing

DNA extracts were used as a template for PCR amplification of 16S rRNA tags (V4 region; 408 nt long) using universal 16S primers predicted to bind to 94.6% of all 16S genes i.e. the forward primer F1, 5'-AYTGGGYDTAAAGNG, (RDP's Pyrosequencing Pipeline: http://pyro.cme.msu.edu/pyro/help.jsp) and reverse primer V5, 5-CCGTCAATTYYTTTRAGTTT-3' (Claesson et al., 2010). The primers incorporated the proprietary 19-mer sequences at the 5' end to allow emulsion-based clonal amplification for the 454-pyrosequencing system. Unique molecular identifier (MID) tags were incorporated between the adaptamer and the target-specific primer sequence, to allow identification of individual sequences from pooled amplicons. The PCR reaction contained 25µl BioMix Red™ (Bioline Reagents Ltd., London, UK), 1µl of each primer (10pmol), 5µl DNA template and nuclease free H_2O to give a final reaction volume of 50µl. PCR amplification was performed using a G-Storm thermal cycler (Gene Technologies, UK). The amplification programme consisted of an initial denaturation step at 94°C for 2 mins, followed by 40 cycles; denaturation at 94°C for 1 min, annealing at 52°C for 1min and extension at 72°C for 1 min. A final elongation step at 72°C for 2 mins was also included. Amplicons were cleaned using the AMPure XP purification system (Beckman

Coulter, Takeley, United Kingdom). The quantity of DNA extracted by the different methods was assessed using the Quant-It™ Picogreen® dsDNA reagent (Invitrogen, USA) used in accordance with the manufacturer's instructions and a Nanodrop™ 3300 Fluorospectrometer (Thermo Fisher Scientific Inc, USA). The ND3300 excites in the presence of dsDNA bound with Picogreen® at 470nm and monitors emission at 525nm.

2.3 High-throughput sequencing and bioinformatic analysis

The 16S rRNA V4 amplicons were sequenced on a 454 Genome Sequencer FLX platform (Roche Diagnostics Ltd, Burgess Hill, West Sussex, UK) according to 454 protocols. Read processing was performed using techniques implemented in the RDP pyrosequencing pipeline (Cole et al., 2009). Sequences not passing the FLX quality controls were discarded, the 454 specific portion of the primer were trimmed, the raw sequences were sorted according to tag sequences and reads with low quality scores (quality scores below 40) and short length (less than 150 bp for the 16S rRNA V4 region) were removed as well as reads that did not have exact matches with the primer sequence. The QIIME suite of programs was used to align, chimera check, cluster and carry out phylogenetics on sequence reads, as well as, to measure microbial α-diversities and to plot rarefaction curves to determine if sequencing was carried out to sufficient depth (Caporaso et al., 2010). β-diversities were calculated on the sequence reads based on Weighted Unifrac and principal coordinate analysis (PCoA) performed. KiNGviewer was used to visualise PCoA plots. Trimmed fasta sequences were assessed using BLAST (Altschul et al., 1990) against the SILVA version 100 database (Pruesse et al., 2007). The resulting BLAST output was parsed using MEGAN version 62.3.0 (Huson et al., 2007). MEGAN assigns reads to NCBI taxonomies by employing the Lowest Common Ancestor algorithm which assigns each RNA-tag to the lowest common ancestor in the taxonomy from a subset of the best scoring matches in the BLAST result. Bit scores were used from within MEGAN for filtering the results prior to tree construction and summarisation (absolute cut-off: BLAST bit-score 86, relative cut-off: 10% of the top hit) (Urich et al., 2008). The statistical significance of differences in proportions of

microbial taxa was determined by the non-parametric Kruskal-Wallis test (Kruskal and Wallis, 1952) using Minitab® statistical package.

2.4 Culturing of *Thermus*

2.4.1 Culture-based Method

Castenholz TYE medium was chosen to selectively support the growth of strains from the genus *Thermus*. Castenholz TYE medium was prepared by mixing 5 parts 2X Castenholz salts with one part 1% TYE and 4 parts distilled water. Castenholz Salts, 2X contained 0.2 g nitrilotriacetic acid, 0.12g $CaSO_4.2H_20$, 0.2g $MgSO_4.H_2O$, 0.016g NaCl, 0.21g KNO_3, 1.4g $NaNO_3$, 0.22g Na_2HPO_4, 2.0ml $FeCl_3$ solution (0.03%) and 2.0ml Nitsch's Trace elements {0.5ml H_2SO_4, 2.2g $MnSO_4$, 0.5g $ZnSO_4.7H_2O$, 0.5g H_3BO_3, 0.016g $CuSO_4.5H_2O$, 0.025g $Na_2MoO_4.2H_20$, 0.046g $CoCl_2.6H_2O$ distilled water 1L}, adjusted to a final volume of 1 L and final pH of 8.2. 1% TYE solution consisted of 10.0 g tryptone, 10.0 g yeast extract dissolved in 1 L distilled water. The final pH of Castenholz TYE medium should be 7.6. For preparation of the corresponding agar, 3% (w/v) bacteriological agar was added to the final solution. *Thermus* was isolated by enriching for 3 days at 70°C in Castenholz medium followed by isolation on Castenholz agar at 55°C for a further 3 days.

2.4.2 PCR and qPCR-based detection of Thermus

A set of primers (TpolFor; 5'-AGCCTCCTCCACGAGTTC-3' and TpolRev; 5'-GTAGGCGAGGAGCATGGGGT-3') targeting a region specifically conserved within the polymerase 1 gene of *Thermus* were designed to facilitate PCR and qPCR-based detection of the genus. The theoretical specificity of these primers was tested using the oligo probe search tools in the BLAST classifier database (Altschul et al., 1990). PCR amplification of the polymerase 1 gene using these primers was carried out under the following parameters: 95°C for 2 min initial denaturation, followed by 40 cycles of 94°Cx30 s, 63°Cx30 s, 72°Cx45 s, and a final elongation of 72°C for 2 min. The resultant products were visualised by agar gel electrophoresis. Amplicons generated were cleaned using the Roche High Pure PCR clean-up kit and sequenced (Source Bioscience; Dublin, Ireland). The specificity of the primer pair was tested

using DNA from a selection of cheese-associated Gram-positive and Gram-negative cultures i.e. *Streptococcus thermophilus* (Defined Starter Mix, TFP, France)*, Lactobacillus helveticus* DPC6865*, Propionibacterium freudenreichii* DPC6451 and *Lactococcus lactis* HP as well as *Escherchia coli, Listeria monocytogenes* EGDe*, Salmonella typhimurium* LT2 and *Bifidobacterium longum*.

To facilitate the quantification of *Thermus* by molecular means, a quantitative real-time (qPCR) protocol was designed. Genomic DNA extracted from *Thermus thermophilus* HB27 (DSMZ Culture Collection) using the PowerFood Microbial DNA extraction kit (Cambio). A PCR product from within the polymerase1 gene was generated using the genus-specific primers, as described above. Purified amplicons were cloned into the pCR®2.1-TOPO vector using the TOPO-TA cloning system (Invitrogen, Life Technologies, Carlsbad, California) in accordance with manufacturer's instructions. Following cloning, the complete vector was transformed into chemically competent TOP-10 *E. coli* cells (Invitrogen) and harvested on LB media containing 100 µg ml^{-1} ampicillin. The accuracy of the cloned amplicon was confirmed by restriction analysis and DNA sequencing. QPCR standards were prepared following the linearization of plasmid DNA with *pst* restriction enzyme and quantification with the Nanodrop ND-1000 (Thermo Fisher Scientific Inc). A standard curve was then generated via a series of dilutions from 10^2 to 10^8 copies $µl^{-1}$ DNA. The LightCycler 480 SYBR Green I Master kit (Roche Diagnostics GmbH, Mannheim, Germany) was used for quantification according to the manufacturer's instructions. Each PCR reaction contained 5µl Sybr green master mix (Roche), 1 µl of both forward and reverse primer (7.5 pmol), 2 µl of DNA and was made up to a final volume of 10 µl with nuclease free dsH_2O. The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 20 sec, annealing at 61°C for 15 sec and elongation 72°C for 20 sec. Assays were performed in triplicate. To facilitate quantification by qPCR, we applied the formula of Quigley et al., 2013, to convert from copies $µl^{-1}$ to cfu g^{-1} of cheese.

2.5 Cheese spiking studies

2.5.1 Cheese manufacture and analysis

The starter cultures *S. thermophilus* (Defined Starter Mix, TPF, France) and *L. helveticus* DPC6865 were sourced from the culture collection at Facility 1. These were each grown overnight at 37°C in reconstituted low heat-skim milk powder, which had first been heat-treated at 90°C for 30 min. *Propionibacterium freudenreichii* DPC6451 was grown for 3 days at 30°C in sodium lactate broth. All starter cultures were obtained from Facility 1 culture collection. *T. thermophilus* DPC6866, obtained from a cheese with a pink defect, was grown in Castenholz broth at 60°C with shaking for 36 hours. Cells were collected by centrifugation at 14,000 *g* for 20 mins, washed once to remove trace media and resuspended in sterile water. Raw milk was obtained from Teagasc, Moorepark dairy herd, standardised, pasteurised at 72°C for 15 sec and pumped at 32°C into four individual cylindrical stainless steel vats with automated variable speed cutters and stirrers. This milk was employed to manufacture Swiss-type cheese at pilot-scale level in Moorepark Technology Ltd (Fermoy, Cork, Ireland). Details with respect to the manufacture of control and test cheeses can be found in Table 2. Enumeration of microbiological content, composition of cheeses and proteolysis were measured at various stages of ripening (Table 3). To enumerate specific bacterial components, cheese samples were aseptically removed, placed in a stomacher bag, diluted 1:10 with sterile tri-sodium citrate (2% w/v, Sigma) and homogenised in a Seward Stomacher® 400 Lab System (Seward Ltd., West Sussex, UK) for 2 min. Further dilutions were prepared as required. Viable *S. thermophilus* were enumerated on M17 agar (Oxoid) with 0.5% lactose (Oxoid) at 42°C for 3 days. *L. helveticus* were enumerated on MRS agar (Oxoid) adjusted to pH5.4 at 37°C for 3 days under anaerobic conditions. PAB levels were enumerated on sodium lactate agar containing 40 µg ml^{-1} kanamycin (Sigma) at 30°C for 7 days under anaerobic conditions. Non-starter lactic acid bacteria (NSLAB) were enumerated on *Lactobacillus* Selective Agar (LBS; Difco) at 30°C for 5 days aerobically. *T. thermophilus* was monitored using qPCR methods. To facilitate this, DNA was extracted from milk, whey or 10 ml cheese homogenate using the PowerFood DNA isolation kit as described above. Grated samples from cheeses were analysed for salt (IDF, 1988), moisture

(IDF, 1982) and protein (IDF, 1993) after 11 days of manufacture, pH (Standards, 1976) was measured throughout ripening. The levels of nitrogen soluble at pH 4.6 (pH 4.6SN) were measured as described by Sheehan et al. (2007). Free amino acid analysis was carried out on pH 4.6SN extract as described by Fenelon et al. (2000)

2.5.2 Visual detection of pinking

Cheese rounds were examined visually throughout ripening for the formation of pink discolouration defect. Pink colour formation was quantified with a Chroma Meter using Hunter, ***L, a, b*** colour scale. The colour was measured using fresh sliced exposed cheese surface. The colour meter was standardised with a white standard plate (Y=88.31, x=0.3160, y=0.3226). Hunter ***a*** (redness) values were recorded.

2.5.3 Statistical Analysis

A randomised complete block design that incorporated the four treatments and 3 blocks (replicate trials) was used for the analysis of response variables relating to the composition of cheeses, moisture, salt and protein, as well as starter bacteria, PAB, NSLAB, *T. thermophilus*, pH, pH4.6SN, FAA and apparent colour differences. Analysis of variance was carried out on data using the general linear model procedure of SAS (SAS Institute). The Tukey honestly significant difference test was used to determine the significance of difference between the means. The level of significance was determined at $p < 0.05$.

2.6 Environmental sampling

Monitoring for the presence of *Thermus* was carried out at Facilities 1 and 2. Samples that were collected included swabs of surface areas covering starter culture vats, milk vats, mixing vats and pressing vats, pasteuriser and water hoses. Liquid samples included water sources, pre- and post- brine solution, as well as brine sock solution. Also assessed were pre- and post- clean-in-process solutions (CIP), antifungal dips and batch starter cultures. For all

samples, culture-based and culture-independent based *Thermus* detection methods were applied as described above.

3. Results

3.1 High-throughput 16S sequencing of samples extracted from cheeses with and without a pink defect

DNA was extracted from control and pink defect samples of three different cheese types i.e. a Swiss-type cheese, a “thermophilic Cheddar”-type cheese and Cheddar cheese with annatto dye. 16S rRNA amplicons were generated and subjected to high throughput DNA sequencing. Given that microbial diversity was expected to be relatively low, ≥3,500 16S reads per cheese was targeted and exceeded (average number of reads per sample was 3960). Rarefaction curve of α-diversity, represented by Shannon indices, for all samples sequenced confirmed that satisfactory coverage was achieved (Figure 1). Principal Co-ordinate Analysis of β- diversity (PCoA), according to Weighted Unifrac distances, revealed the existence of two clusters of microbial populations (Figure 2). Cluster I is associated with the annatto-type cheeses, while cluster II contains the Swiss-type and Cheddar-type cheeses. Within cluster II there appears to be a further split in the community, whereby the data points representing the Swiss-type defect samples, in general, cluster away from other data points in cluster II.

3.2 Identification of bacteria by pyrosequencing

Phylogenetic analysis of the 16S rRNA reads established that those from the Swiss-type and Cheddar-type cheeses corresponded to five different bacterial phyla (Figure 3a), i.e. Firmicutes, Proteobacteria, Bacteroides, Actinobacteria and Deinococcus-Thermus, whereas the annatto-coloured cheeses contained Firmicutes only. Firmicutes also dominated in the two cheese types. In Swiss-type cheese, these corresponded to 99% and 94% of assigned reads in control and defect cheeses, respectively. Similarly, in Cheddar-type cheeses, Firmicutes corresponded to 97% and 93% of assigned reads in the control and defect cheeses. Deinococcus-Thermus reads were detected in the defect-associated Swiss-type cheese (6%) only. In Cheddar-type cheeses, the number of reads assigned to this phylum was significantly higher (7%) than in control cheeses (3%; $p=0.004$). In these two cheese types, reads corresponding to other phyla corresponded to less than 1% of assigned

reads. When these reads were assigned at the family level, eleven families were identified (Figure 3b). *Streptococcaceae* (38-31%; 79-69%; 95-93%) and *Lactobacillaceae* (61-61%; 17-23%; 5-7%) dominated in all three cheese types (control-defect samples of Swiss-, Cheddar-type and annatto-coloured cheeses, respectively). Proportions of *Thermaceae* were equal to those of the corresponding *Deinococcus-Thermus* phylum i.e. 6% and 0% in defect and control Swiss-type cheeses, respectively, and 7% and 3% in defect and control Cheddar-type cheeses, respectively. When these reads were assigned to genus level, 15 genera were identified (Figure 3c). *Streptococcus* and *Lactobacillus* dominated in all cheese types. Proportions of the genus *Thermus* matched the previously assigned Deinococcus-Thermus and *Thermaceae* and thus were significantly greater in Swiss-type and Cheddar defect cheeses (p=0.002 and 0.004, respectively). Lactococci were also present in cheddar-type cheeses at low levels (1-2%). Annatto-coloured cheeses contained high levels of *Lactococcus*, 28% and 26%, in control and defect cheeses, respectively. No statistically significant differences in proportions of different taxa were observed between control and defect annatto-coloured cheeses.

3.3 Detection of *Thermus* in Cheese

Following the identification of reads assigned to the genus *Thermus* in the Swiss-type and Cheddar-type cheese, and in particular its association with samples of cheeses containing the pink discolouration defect, attempts were made to isolate this bacterium, which is not regarded as being a typical cheese-associated genus, from the defect cheeses. Castenholz medium was employed as it has previously been shown to support the growth of *Thermus* (Brock and Freeze, 1969) but, due to its minimal nutrient content, was unlikely to support the growth of other genera. An enrichment step, whereby cheese was homogenised in Castenholz medium and incubated at 70°C for 3 days, was employed to encourage the growth of *Thermus*, which are characterised by their highly thermophilic nature, and to prevent the growth of more moderately thermophilic cultures such as those within the starter culture population. A 3% agar was employed to allow incubation at high temperature

(55°C) without rapid dehydration of the media. Use of this approach resulted in the successful isolation of *Thermus* from defect cheese.

Rapid, culture-independent PCR-based methods to detect *Thermus* were also developed. A primer pair was designed with a view to selectively amplifying the polymerase I gene of *Thermus* and assays with a broad variety of controls established the primers to indeed by specific. To take full advantage of the specificity of these primers, a corresponding qPCR-based protocol was developed. Use of this approach, together with template DNA from the cheeses that were the subject of high-throughput sequencing, resulted in the level of *Thermus* present being quantified. As expected, Swiss-type control cheese contained no *Thermus*, while the defect samples contained on average 1.77 x 10^3 cfu g^{-1}. Cheddar-type cheese contained 3.87x10^1 and 2.12x10^3 cfu g^{-1}, for control and defect cheeses, respectively.

To confirm the presence of *Thermus*, from culture-based and PCR-based assessment of the cheeses, we carried out PCR using the *Thermus*-genus specific primers, as described above. Purified amplicons were sequenced and assigned as *Thermus thermophilus* DPC6866.

3.4 Formation of "pinking" in spiked cheese

To establish definitively that *Thermus* is responsible for the formation of pink defects in cheese, we carried out a trial whereby we produced cheese containing *T. thermophilus* and compared pink development relative to that of a control cheese. For this, we manufactured a Swiss-type cheese. Trials were carried out in triplicate and in each instance four cheeses were produced. These included the control cheese, which contained no *T. thermophilus*, and three experimental cheeses, all of which contained *T. thermophilus* at 10^6 cfu g^{-1}. Experiment 1 (exp 1) cheese contained *T. thermophilus* with starter cultures at normal levels (500 g L. helveticus, 250 g S. thermophilus, 4 g PAB). Experiment 2 (exp 2) cheese contained *T. thermophilus* with higher levels of *L. helveticus* (500 g), Finally, experiment 3 (exp 3) cheese contained *T. thermophilus* with higher levels of *L. helveticus* (500 g) and lower levels of *S. thermophilus* (250 g) (Table 2). The reasoning behind the varying levels of *L. helveticus* and *S. thermophilus* was due to the increased and decreased

levels of these bacteria (respectively), as detected by the pyrosequencing data where *Thermus* was present.

3.4.1 Starter, PAB and NSLAB viability during cheese ripening

Mean viable cell numbers of *S. thermophilus* were determined to be 10^7 cfu g^{-1} at day 1 of ripening in control, exp 1 and exp 2 cheeses and at 10^6 cfu g^{-1} in exp 3 cheese, which correlates with levels of starter *S. thermophilus* inoculated into the cheese milk. There were significant increases (p=0.0063) in the numbers of *S. thermophilus* over time (Figure 4) but there were no significant differences between treatments. *L. helveticus* numbers were 1 x 10^6 cfu g^{-1} at 1 d ripening, in control and exp 1 cheese, while exp 2 and exp 3 cheese contained 5 x 10^6 cfu g^{-1}, again reflecting the different levels of *L. helveticus* starter added (Figure 4). The changes observed in levels of *L. helveticus* during cheese production were not significant. Counts of PAB increased significantly until 46 d ripening (p<0.0001) (Figure 4), however they did not differ significantly between treatments. Viable NSLAB numbers increased significantly until the end of warm room ripening (Figure 4) (p<0.0001). We observed a significant difference in the levels of NSLAB between control cheese and exp 2 cheese (p=0.0438) and control cheese and exp 3 cheese at 60 d ripening (p=0.0225).

3.4.2 Survival of Thermus thermophilus throughout cheese manufacture and ripening

Thermus thermophilus was inoculated with a view to obtaining >10^4 cfu g^{-1} in the three experimental cheeses. Using culture-independent qPCR, we determined the levels of *T. thermophilus* present in the inoculated milk, lost in whey, and retained in curd, as well as throughout ripening (Figure 5). We established that *Thermus* was present at 10^6 cfu ml^{-1} in milk after 1 h inoculation (sampled prior to rennet addition). There was some loss of *T. thermophilus* in whey, i.e. 10^2 cfu ml^{-1}, however, considerable levels were retained within the curd (10^5 cfu g^{-1}). Control cheeses, which were not spiked with *T. thermophilus*, were also assessed and were found not to contain *Thermus* (data not shown), establishing that no natural contamination, or cross-contamination, occurred during production. Slight numerical increases

in the levels of *T. thermophilus* were noted during hot room ripening, however these were not significant. Following transfer to the cold room for continued ripening, we observed a slight decrease in the levels of *T. thermophilus* to 10^4 cfu g^{-1}. This was consistent across all three experimental cheeses (Figure 5).

3.4.3 Composition of cheeses

The gross composition of cheeses at 11 d ripening was assessed and is summarised in Table 5. All cheeses had statistically similar pH values, levels of moisture, salt distribution and protein. The consistency of these results between cheeses and cheese trials indicate good repetition across each day of manufacture i.e. no significant differences were detected between these variables. Significant increases in pH (Figure 6), pH 4.6SN (soluble nitrogen) (Figure 7) and total FAA (p<0.0001 for all three parameters assessed) were observed throughout ripening. The concentrations of individual FAAs (mg kg^{-1} of cheese) in all cheeses at 116 d of ripening are shown in Figure 8. The FAAs present at greatest concentrations in the cheeses at most ripening times were glutamic acid, valine, leucine, lysine and proline, and were in line with that expected in Swiss-type cheeses (Sheehan et al., 2008).

3.4.4 Visual Examination for "pinking" formation in cheese

To quantify the formation of "pinking" in the cheese samples we applied a Chroma Meter using Hunter ***L, a, b*** colour scale throughout ripening. Here, we report Hunter ***a*** values, which determines the level of redness (+) to greenness (-) (Wadhwani and McMahon, 2012). Changes in the ***a*** values are summarised in Table 6. Here, we observe that the ***a*** reading is a negative value, establishing that the overall colour is in the green spectrum. However, throughout the centre of the experimental cheeses there is a shift towards a more positive value. These differences were first noted after 116 d ripening (***a*** = -2.08, -1.91, -1.75, -1.73, for control, exp 1, exp 2 and exp 3 cheeses, respectively) and the intensity of this value and the formation of a pink hue developed further in exp 2 cheese at 144 d, (***a***=-.2.38 -1.95, -1.34 and -1.82 for control, exp 1, exp 2 and exp 3 cheeses, respectively). This further pinking of exp 2 cheese between 116 d and 144 d was statistically significant

(p=0.0108). The exp 2 144 d values were also significantly less negative than those of the control (p=0.0009) and exp 1 cheeses (p=0.0235).

3.5 Thermus is present at a number of locations in dairy processing plants

To investigate how *T. thermophilus* becomes part of the cheese population, an environmental screen of Facility 1 and Facility 2, whereby swab and liquid samples were collected from around the plant, was undertaken (Table 4). The presence of *Thermus* in these samples was assessed using both culture-dependent and qPCR methods. While *Thermus* was detected in a number of samples, its presence was most consistently detected in hot water sources (Table 4). Notably, *Thermus* is a known thermophillic water bacterium and has been isolated previously from hot tap water (Pask-Hughes and Williams, 1975).

4. Discussion

Here, high-throughput DNA sequencing of 16S rRNA amplicons was performed to profile the bacterial content of cheeses displaying a pink discolouration defect and of controls. Three types of cheeses with which this problem has been associated, including Swiss-type cheese, “thermophillic”-Cheddar type cheese and Cheddar cheese with annatto dye, were assessed.

Firstly, we noted that Cheddar cheese with annatto dye was populated with typical cheese bacteria only and no significant differences were observed between the defect and control cheeses. This is consistent with the literature which has indicated that the cause of pink discolouration in this type of cheese is due to physiological, rather than microbiological factors. It is suggested that a decrease in cheese pH (<pH5.4) may results in precipitation of norbixin (a component of annatto) leading to a pink discolouration. It has also been suggested that Maillard browning, or enzymatic conversion of tyrosine to melanin pigments, may result in discolouration of this cheese type (Daly et al., 2012).

Sequencing of the microbiota of Swiss-type and “thermophillic”-type Cheddar cheeses revealed a difference in the bacterial content of these cheeses. Specifically, the presence of a phylum not previously regarded as a major component of the cheese microbiota, Deinococcus-Thermus, at significantly higher levels in defect cheese types (p=0.002 and 0.004, respectively). Bacteria from the phylum Deinococcus-Thermus are known for their resistance to extreme stresses, including radiation, oxidation, desiccation and high temperature. Cultured Deinococcus-Thermus usually have a red or yellow pigment because of their ability to synthesize carotenoids (Tian and Hua, 2010). Interestingly, members of this phylum, *Deinococcus* species and *Meiothermus* species, have been associated with pink hue formation in various environments, including discoloured paper in paper manufacture industries (Ekman et al., 2007, Kolari et al., 2003). Also, an ancient terrace, referred to as “The Pink Terraces” which were recently re-discovered by geoscientists in New Zealand (Woods Hole Oceanographic Institution, MA, USA), emit a pink hue most likely due to the presence of *Thermus ruber* bacterium (The Encycleopedia of New Zealand). Further analysis of the DNA

sequence data determined that at genus level, these sequences are assigned to the bacterium *Thermus*. Subsequent PCR assays with an isolated strain allowed us to determine this to be *Thermus thermophilus*. *T. thermophilus* is a gram negative, extremely thermophilic, aerobic microorganism (Tian and Hua, 2010). It has been associated strongly with hot water sources, including hot springs (Pěčková, 1991, Sharp and Williams, 1988), as well as hot tap water (Pask-Hughes and Williams, 1975), which, on the basis of our environmental monitoring, is a potential point of entry into the cheese manufacturing process.

Following the detection of this bacterium and its strong association with cheeses with pink defects, we further tested our hypothesis that the presence of *T. thermophilus* bacterium is responsible for the pink discolouration in cheese through a series of cheese trials. Here we inoculated cheese with *T. thermophilus*, which was isolated from a Swiss-type defect cheese and also varied the levels of the starter bacteria inoculated into these cheeses. These variations were prompted by our DNA sequence data which revealed that defect Swiss-type cheeses also contained lower levels of *Streptococcus* and a higher level of *Lactobacillus*. Following production of the cheeses, no differences were noted in the chemical composition of the various cheeses. This is consistent with previous studies which also failed to find a correlation between cheese compositional profiles, including profiles relating to moisture, salt, soluble nitrogen and FAAs, and the development of the pink defect (Betzold, 2004, Chang et al., 1985, Martley and Michel, 2001, Shannon et al., 1968). Through an assessment based on colour meter analysis, and from our own visual examination, we determined a significant difference in the levels of “pinking” in the cheeses in which *T. thermophilus* is present. Notably, in situations where the levels of starter cultures were adjusted, particularly where *L. helveticus* was increased, the pink colour formation was much more intense. It has been widely reported that certain strains of starter cultures used for cheese manufacture can be more frequently associated with discolouration in cheese (Bottazzi et al., 2000, Park et al., 1967, Shannon and Olson, 1969), most notable lactobacilli and propionic acid bacteria. Following identification of *T. thermophilus* and its association with pink discolouration in cheese, we conducted subsequent analysis of dairy processing plants which

determined its presence in the dairy facilities, particularly with hot water. Our results established that *T. thermophilus* plays a major role in the formation of pink discolouration in cheese, and that activity of starter cultures may have an important factor in the intensity of this development.

5. Conclusion

This is the first occasion that *T. thermophilus* has been detected in a pink discoloured cheese, most likely due to its extreme growth requirements. However, the use of high-throughput sequencing has allowed us to detect its presence. We have been able to determine that hot water entering the cheese facilities is the most likely source of this bacterium. Using spiked cheese trials we have established the relationship between the presence of this bacterium in cheese and the formation of pink discolouration. Through these trials we have determined that *T. thermophilus* associates with the curd, with little loss in whey. We also confirmed that it does not affect the composition of cheese. Once in the cheese, the levels remain quite consistent throughout ripening. The formation of pink discolouration associated with this bacterium appears to occur following hot room ripening. The ability of this bacterium to produce carotenoids is likely associated with the formation of this discolouration, however this remains unproven. Pink discolouration defect in cheese still remains a complex issue; it is more likely that a set of conditions rather than one single factor is required for its development. However the work carried out during this study has revealed that the presence of *Thermus thermophilus* plays a major role in the formation of this defect.

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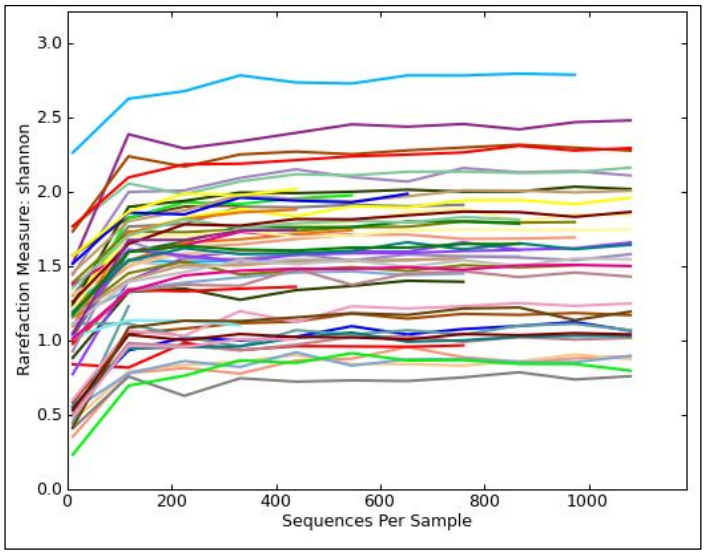

Figure 8: Rarefaction curve of Shannon diversity indicating satisfactory coverage of all samples sequenced.

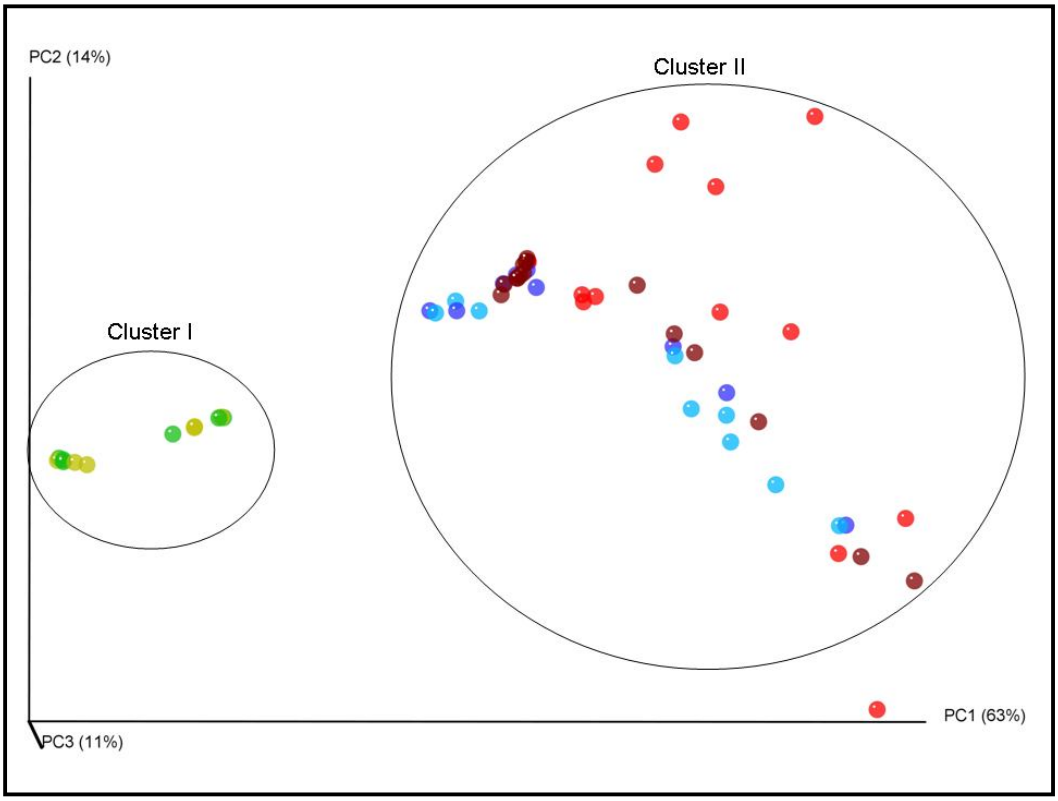

Figure 9: Principal Co-ordinate Analysis (PCoA) plot based on Weighted Unifrac, highlighting a split of the bacterial population into two clusters. Cluster I contains annatto-coloured Cheddar cheeses represented as dark green (control) and light green (defect). Cluster II consists of Swiss-type cheese represented as dark red (control) and bright red (defect) and “thermophilic-Cheddar”-type cheese represented in dark blue (control) and light blue (defect).

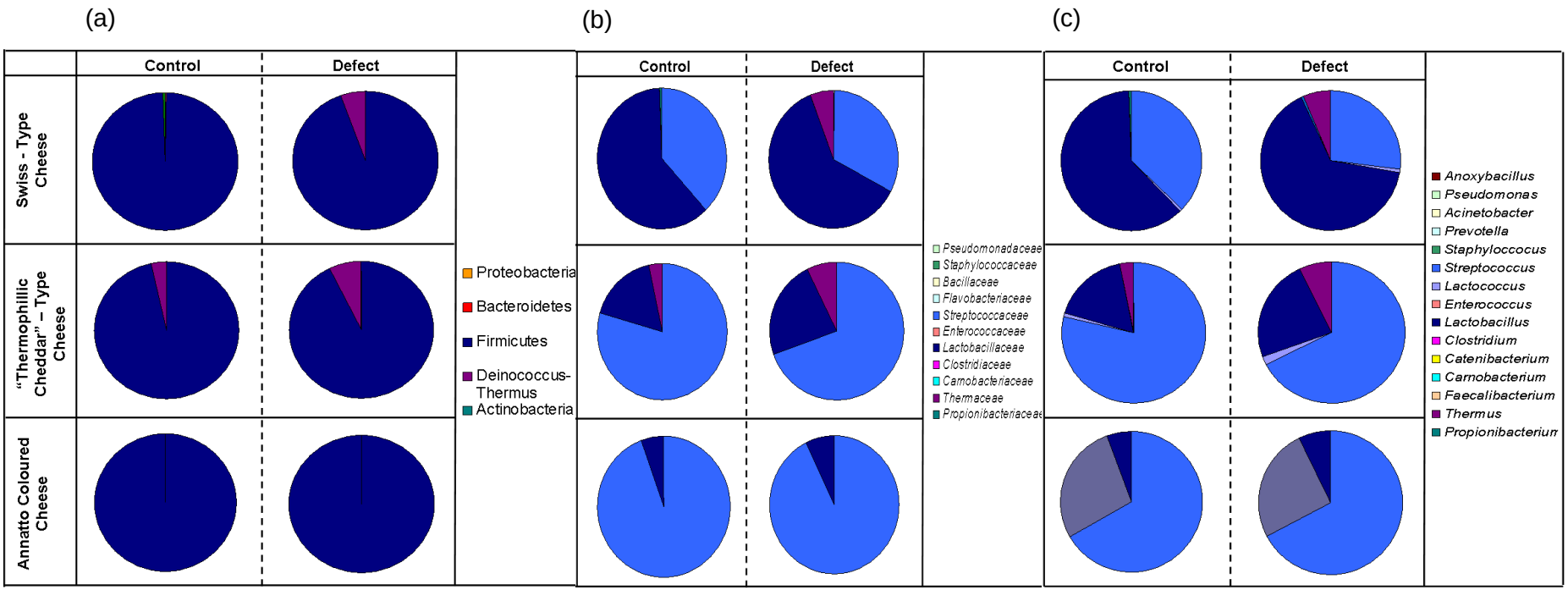

Figure 10: Bacterial composition of defect and control cheeses as determined by high throughput sequencing. 16S rRNA sequences assigned according to MEGAN using the Silva database at the (a) phylum, (b) family and (c) genus levels with the three cheese types affected by the pink discolouration defect and controls populations.

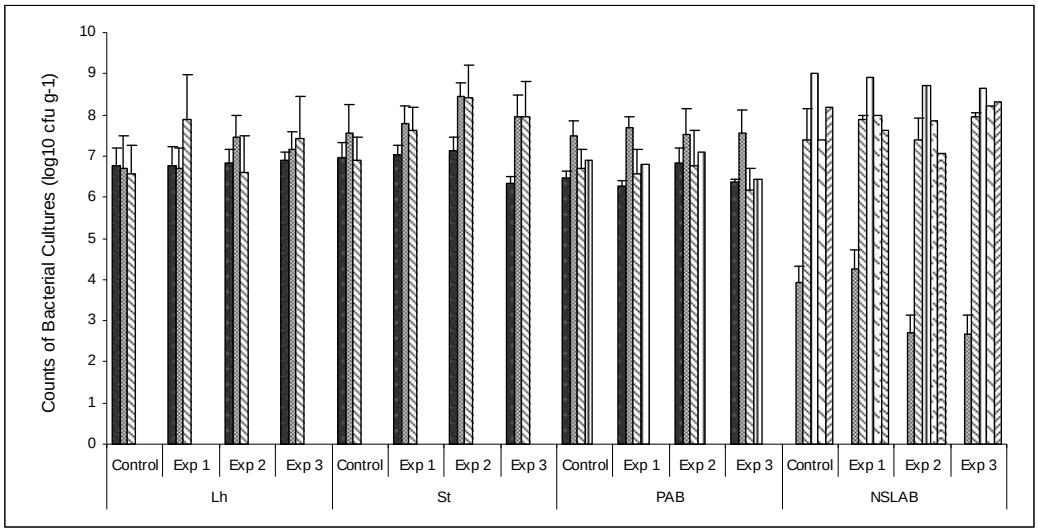

Figure 11: Counts of ripening bacteria, *Lactobacillus helveticus* (Lh), *Streptococcus thermophilus* (St), propionic acid bacteria(PAB) and non-starter lactic acid bacteria (NSLAB) throughout ripening, 1d , 11d , 46 d , 60 d , 88 d , 116 d .

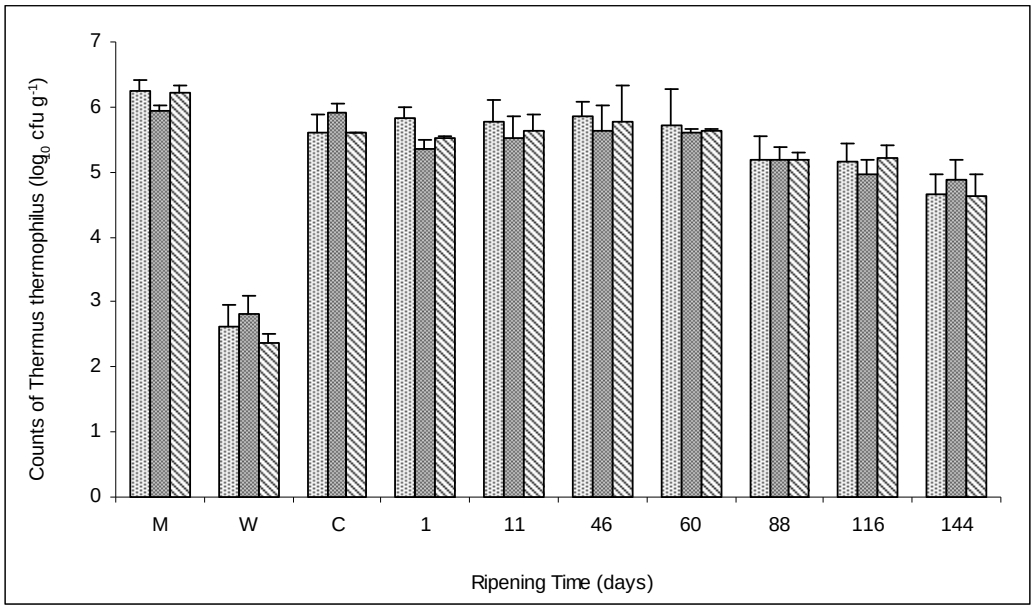

Figure 12: *Thermus thermophilus* levels, as determined by qPCR, throughout manufacture.

M-inoculated milk, W-whey, C-curd and ripening. Experimental chees 1 experimental cheese 2 , experimental cheese 3 .

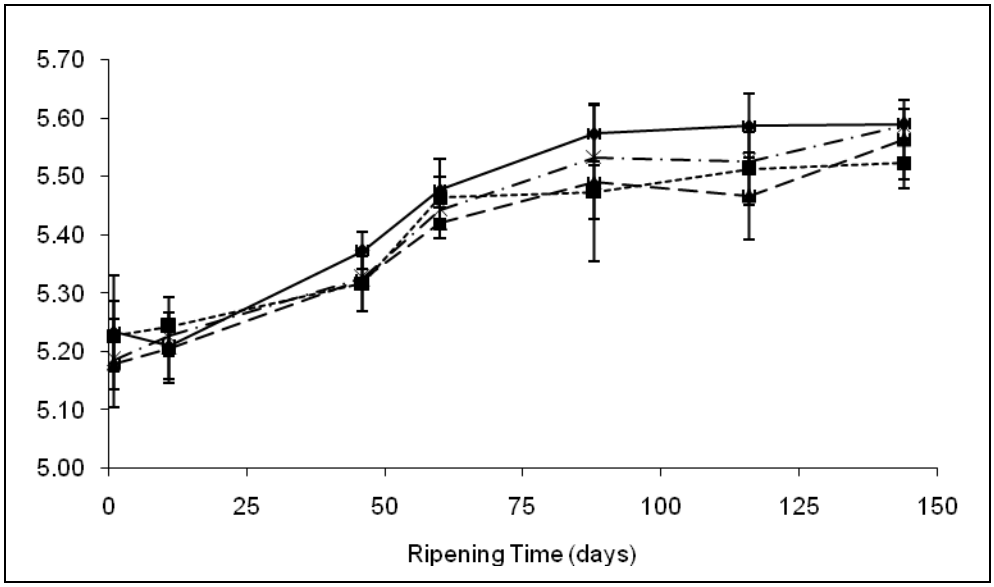

Figure 13: The effect of different treatments on cheese pH over ripening. Control cheese —◆—, experiment 1 cheese ---■---, experiment 2 cheese —▲- - and experiment 3 cheese —×—.

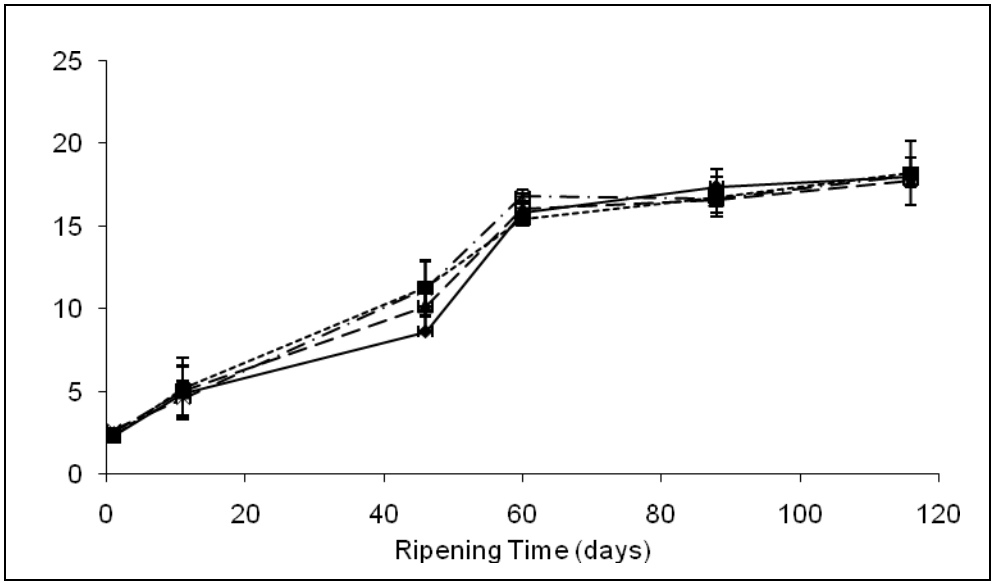

Figure 14: The effect of different experimental set-up on cheese % pH4.6SN over ripening.

Control cheese —◆—, experiment 1 cheese ---■---, experiment 2 cheese —▲- - and experiment 3 cheese —×—.

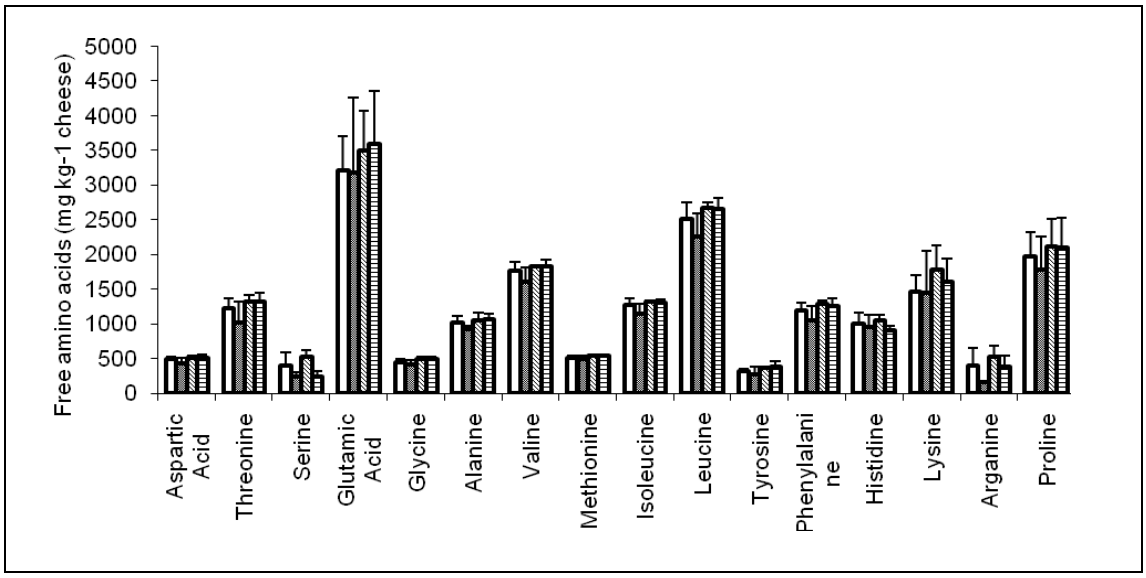

Figure 15: The effect of different experimental set-up on free amino acid levels after 116 days ripening. Control cheese □, experiment cheese 1▩, experiment cheese 2▧, experiment cheese 3▤.

Table 8: Types of cheeses associated with pink discolouration defect and how the defect manifests in each type.

Cheese-Type	Appearance of pink discolouration	Cheese Source
Swiss-Type cheese	a uniform pink border occurring below the external surfaces	Facility 1
"Thermophillic-Cheddar"-type cheese	at the surface of the cheese block	Facility 2
Cheddar cheese with annatto dye	sporadically distributed within the cheese block	Facility 3 and 4

Table 9: Details and differences between manufacture of Swiss-type spiked cheese trials.

Treatment	**Control Cheese**	**Experiment 1 Cheese**	**Experiment 2 Cheese**	**Experiment 3 Cheese**
Milk Volume	454 kg	454 kg	454 kg	454 kg
Starter Culture (w/v)				
Streptococcus thermophilus	500 g	500 g	500 g	250 g
Lactobacillus helveticus	250 g	250 g	500 g	500 g
Propionibacterium freudenreichii	4 g	4 g	4 g	4 g
Test Bacterium cfu ml^{-1}				
Thermus thermophilus	0	10^6	10^6	10^6
Curd Formation	As Standard			
Cook	0.5°C min to 45°C 1°C min to 53°C			
Drain pH	pH 6.30			
Curd Handling	Pre-press and mould			
Salting Method	Brine			
Cheese Size	10kg			
Cool Room Ripening	8.5°C x 10 days			
Hot Room Ripening	22°C x 7 weeks			
Ripening Regime	4.5°C after hot room step			

Table 10: Assessment carried out at different stages of manufacture and ripening.

Ripening Time (days)	Stages of Ripening	Sample Type	Microbiological Analysis	Compositional Analysis
0	Day of manufacture	Milk, Wey, Curd	Tt	pH
1	After Brining	Cheese	Tt, St, Lh, PAB	pH, Moisture, Salt, Proteins, pH4.6SN, FAA
11	After 10 days at cool room ripening (8.5°C)	Cheese	Tt, St, Lh, PAB, NSLAB	pH, Moisture, Salt, Proteins, pH4.6SN, FAA
46	After 5 weeks at warm room ripening (22°C)	Cheese	Tt, St, Lh, PAB, NSLAB	pH, pH4.6SN, FAA, visual examination
60	End of warm room ripening (22°C)	Cheese	Tt, PAB, NSLAB	pH, pH4.6SN, FAA, visual examination
88	After 1 month in cold room (4.5°C)	Cheese	Tt, NSLAB	pH, pH4.6SN, FAA, visual examination
116	After 2 months in cold room (4.5°C)	Cheese	Tt, NSLAB	pH, pH4.6SN, FAA, visual examination
144	After 3 months in cold room (4.5°C)	Cheese	Tt	pH, visual examination

Tt – *Thermus thermophilus*; St – *Streptococcus thermophilus*; Lh – *Lactobacillus helveticus*; PAB – Propionic Acid Bacteria; NSLAB – Non-starter lactic acid bacteria; pH4.6SN – pH4.6 soluble nitrogenFAA – Free Amino Acid.

Table 4: Environmental monitoring of *Thermus* in dairy processing plants.

Area Samples	Sampling Style	Culturing	PCR
Milk Vat	Swab	-	-
Starter Culture Vats	Swab	-	-
Press Vat	Swab	+	-
Pasteuriser	Swab	-	-
Starter Cultures	Liquid	-	-
Hot Water 1	Liquid	+	+
Hot Water 2	Liquid	+	+
Hot Water 3	Liquid	+	+
Brine Before Filter	Liquid	-	-
Brine After Filter	Liquid	-	-
CIP (Before)	Liquid	-	-
CIP (After)	Liquid	-	-
Antifungal Dip	Liquid	-	+

Table 5: Composition of cheeses at 11 days post manufacture.

	pH	% Moisture	% Salt	% Protein
Control	5.21	41.10	1.36	24.931
Exp 1	5.24	40.80	1.25	25.271
Exp 2	5.21	41.50	1.22	25.723
Exp 3	5.23	40.94	1.28	24.804

Data presented in this table are means for three replicate trials.

Table 6: Effect of treatment on colour properties as determined by Hunter ***L, a, b***, dimensions.

Cheese Sample	Area Assessed	a* value	
		116 d	144 d
Control	Top	-2.22	-2.22
	Side	-2.20	-2.17
	Base	-2.01	-2.32
	Centre	-2.08	-2.38
Exp 1	Top	-2.20	-2.21
	Side	-2.05	-2.28
	Base	-2.23	-2.21
	Centre	-1.91	-1.95
Exp 2	Top	-2.57	-2.18
	Side	-2.20	-2.16
	Base	-2.52	-2.10
	Centre	-1.75	-1.34*
Exp 3	Top	-2.08	-2.14
	Side	-1.91	-2.35
	Base	-1.75	-2.13
	Centre	-1.73	-1.82

Here we represent **a** values which indicate formation of redness colour. The results are those taken from 144 d old cheeses

* Statistically significant difference compared to control cheese $p = 0.0009$.

Data presented in this table are means for three replicate trials.

Chapter V

Identification of single nucleotide polymorphisms in strains related to *Lactobacillus helveticus* DPC4571

Abstract

Three strains of *Lactobacillus helveticus*, that are related to the genome sequenced DPC4571 strain, were subjected to high-throughput sequencing to identify single nucleotide polymorphisms (SNPs) that may explain their differing abilities to grow in modified MRS broth and reconstituted skim milk (10% RSM), as well as with different carbohydrate substrates. These strains included two derivatives of DPC4571, which have previously been found to differ with respect to the number of IS elements present, i.e. one contained 18 ISL2 elements (ISL2+) while the other had 15 ISL2 elements (ISL2-), which contrasts with the 17 ISL2 elements in DPC4571. The third strain investigated was *L. helveticus* DPC5607, a close relative of DPC4571. Using genome comparison tools to analyse DNA sequence data generated using the Illumina Genome Analyzer II platform, we identified the presence of a number of SNPs occurring throughout the genome, including in protein-coding genes involved in essential biological functions, such as transport of cellular components, cell structure and function as well as enzymes likely to be involved in important flavour pathways during cheese ripening. A number of other SNPs were located in hypothetical proteins and mobile elements as well as in intergenic regions, which may also impact on the function of nearby genes. These SNPs are likely to contribute to the differing phenotypes of these strains.

1. Introduction

The name *Lactobacillus helveticus* was first given to an isolate from Emmental cheese by Orla Jensen in 1919 (Naser et al., 2006). This Gram-positive, homofermentative lactic acid bacterium (LAB) is one of the species most commonly used in the production of fermented milk beverages and some hard cheeses. It is notable by virtue of its ability to grow at a relatively high temperature (~55°C) and its ability to utilise a wide variety of protein sources thanks to its highly efficient proteolytic system and intracellular peptidases (Slattery et al., 2010). One such strain, *L. helveticus* DPC4571, which was isolated from cheese whey, was selected for genome sequence analysis (Callanan et al., 2008). Other genome sequences of *L. helveticus* include strain H10, an isolate of fermented milk in Tibet (Zhao et al., 2011), strain DSM20075, an Emmental cheese isolate (direct sequence submission), strain R0052 isolated from sweet acidophilus milk (Tompkins et al., 2012) and strain MTCC5463, a vaginal tract isolate (Prajapati et al., 2011). These have been the focus of an extensive review by Cremonesi et al. (2012). The interest in DPC4571 stemmed from the fact that, when used as an adjunct starter culture, it contributed to the production of cheese of consistently high quality. This was attributed to its rapid autolysis and high proteolytic activity (Slattery et al., 2010). At that point genome comparisons demonstrated that its nearest genome sequenced comparator was the gut microorganism *L. acidophilus* NCFM, which suggested that the difference between these dairy and gut lactobacilli is due to relatively few but highly specific gene sets (Callanan et al., 2008). These changes appear to have resulted from deletions, non-sense mutations and truncations within the *L. helveticus* genome. It was also found that DPC4571 encodes 213 highly diverse insertion sequence (IS) elements. The number of IS elements is approximately 10 times more than is found in other lactobacilli and consists of 21 different groups of IS elements. It is thought that these elements have contributed to the molecular evolution of the *L. helveticus* species (Callanan et al., 2008). Previous studies by Kaleta (2010); unpublished) focused on two derivatives of *L. helveticus* DPC4571 which differed with respect to copies of the IS elements, ISL2, which is present in 17 copies in DPC4571. One

derivative, ISL2+, contained eighteen complete copies of the ISL2 element, while the other, ISL2-, contained fifteen complete copies of the ISL2 element. Differences in the growth ability of these two derivatives in complex media and reconstituted skim milk have been noted previously (Kaleta, 2010). Here, we carry out a more detailed genotypic and phenotypic analysis of ISL2+, ISL2- and of a closely related third strain, *L. helveticus* DPC5607 (Kaleta et al., 2009) and compare the results with corresponding DPC4571 data.

2. Materials and Methods

2.1 Bacterial Isolates

Dairy isolate *L. helveticus* DPC4571 as well as two derivatives, which differed from this strain with respect to the number of ISL2 elements i.e. ISL2+ (18 elements) and ISL2- (15 elements), and another dairy isolate, *L. helveticus* DPC5607, were obtained from the Teagasc Food Research Centre Culture Collection. All strains were grown in modified MRS (mMRS) containing 0.5 g L^{-1} L-cysteine (Sigma) at 37°C overnight under anaerobic conditions, unless stated otherwise.

2.2 Carbohydrate Utilisation

Carbohydrate substrate utilization profile analysis was carried out using the API 50 CHL system (BioMerieux, Marcy l'Etoile, France). Overnight cultures were standardised to obtain a final OD_{600} of 2.0. Cells were harvested by centrifugation at 12,000 *g* x 5 min and resuspended in 2 ml of API 50 CHL medium. From this solution, a 25-fold dilution was prepared in sterile maximum recovery diluent (MRD) (Oxoid), 100 µl of which was used to inoculate the API 50 CHL strips. The strips were incubated at 37°C for 48 h under anaerobic conditions after which time results were determined.

2.3 Relative growth and acidifying activity of *L. helveticus* strains

The growth of *L. helveticus* DPC4571, ISL2+, ISL2- and DPC5607 in mMRS broth was measured spectrophotometrically over 24 h at OD_{600}. To evaluate milk acidifying activity, the change of pH in 10% reconstituted skim milk (RSM) inoculated with the respective lactobacilli was recorded every 2 h over a 24 h period. The two media types were inoculated with a 1% inoculum from fresh overnight cultures. In each case, studies were carried out in triplicate.

2.4 Whole genome sequencing, sequence alignments and genome comparisons

Genomic DNA was isolated from overnight cultures of *L. helveticus* ISL2+, *L. helveticus* ISL2- and *L. helveticus* DPC5607 according to Hoffman and Winston (1987). The DNA preparation, at a concentration of 5 µg, was

submitted for high-throughput DNA sequencing using paired-end sequencing on an Illumina Genome Analyser II platform (Sequencing Centre, University College Dublin, Dublin, Ireland). Prior to sequence alignment, quality checks were carried out on raw sequence data using the FastQC program v0.10.1. All DNA sequence data were aligned to the *Lactobacillus helveticus* DPC4571 reference genome (Callanan et al., 2008) using Burrows Wheeler Aligner (BWA) program (Li and Durbin, 2009). Sequences were aligned, sorted and filtered for possible PCR duplicates using Samtools v0.1.17 (Li et al., 2009). DNA sequence variants were identified for each sequence using Samtools v0.1.17. Variant calling was carried out using VarScan 2.2 (Koboldt et al., 2009) with the following parameters, minimum coverage (30), min reads2 (8), min-ave-qual (20) and p-value (0.01). Whole genome comparisons to highlight the presence of single nucleotide polymorphisms (SNPs) were visualised by manual inspection against the reference strain using BamView (Carver et al., 2010) and for gene location using Artemis with BamView (Carver et al., 2013).

3. Results

3.1 Phenotypic differences between *L. helveticus* ISL2+, ISL2-, DPC5607 and the reference DPC4571 strain

The growth of *L. helveticus* ISL2+, ISL2-, DPC5607 and DPC4571 in complex medium (mMRS), their relative ability to acidify 10% RSM and their carbohydrate utilisation profiles were tested. Differences were noted with respect to the growth of the 4 strains in mMRS and their relative ability to acidify RSM (Figure 1). More specifically, DPC4571 grew more effectively in mMRS and more rapidly acidified 10% RSM than the three strains. It was noted that the behaviour of ISL2+ and ISL2- varied depending on the growth medium used i.e. when grown in complex mMRS medium, ISL2+ had the relatively greater growth rate (Figure 1(a)), while ISL2- grew more effectively in RSM medium, on the basis of a relatively more rapid acidification (Figure 1(b)). While the initial growth of DPC5607 in mMRS was not as rapid as that of DPC4571, it did ultimately reach similar optical density levels by stationary phase. The acidification of RSM by DPC5607 followed a pattern that was similar to that of DPC4571 but at a marginally slower rate (Figure 1).

Analysis of the carbohydrate fermentation patterns of the four strains using API 50 CHL-based biochemical testing also revealed differences (Figure 2). We observed that DPC4571 is capable of fermenting glucose, mannose, N-acetyl glucosamine, cellobiose and sucrose (Figure 2(a)). The profiles of the ISL2 derivatives were identical but differed from that of DPC4571, in that both utilised glucose, mannose and esculine ferric citrate (Figure 2(b) and 2(c)). The DPC5607 strain on the other hand only utilised mannose and esculine ferric citrate (Figure 2(d)). Surprisingly, the strains did not utilise lactose, the primary carbohydrate source for *L. helveticus* in dairy environments. It may be that the cell numbers used for API-based investigations are too low to metabolise sufficient lactose to provide a positive result.

3.2 Genome sequence analysis and SNP detection

Genome sequencing of *L. helveticus* ISL2+, *L. helveticus* ISL2- and *L. helveticus* DPC5607 was carried out. Two files were assigned to each strain representing each paired-end run. Sequencing quality was checked in each

case using FastQC which established that the DNA sequence data was of a high quality across all bases (Figure 3(a)) with all scores above 20. Also, average quality per read demonstrated high quality distribution (Figure 3(b)). All other parameters assessed passed quality checks, including sequencing content across all bases, GC distribution over all sequence lengths, GC content across all bases, N content across all bases, distribution of sequence lengths over all sequences, sequence duplication level and relative enrichment over read length (data not shown).

Following alignment to the reference strain, SNPs were visualised using BamView and for gene location using Artemis with BamView. The application of BamView allows determination of the presence of SNPs. Following the removal of SNPs likely to be due to sequencing errors, true SNPs were subjected to further analysis.

3.3 SNP occurrence in ISL2+, ISL2- and DPC5607

We detected the presence of 46, 47 and 88 SNPs for ISL2+, ISL2- and DPC5607, respectively. The SNPs were distributed throughout the genome and could be classed, across all SNPs detected, into those located within genes (26%), those detected in intergenic regions (42%) and those detected in pseudo genes (12%). SNPs occurring within genes (Table 1) are distributed between genes of known function, hypothetical proteins and mobile elements. We observe that many of the SNPs present are common to three of the newly sequenced strains.

3.3.1 SNPs within genes of known function

The genes of known function containing SNPs were most frequently those associated with transport, transcription and DNA synthesis (Table 1). SNPs were found in the genes encoding ABC transporter ATP binding protein, ATP dependent DNA helicase DinG, cell division protein FtsZ, transcriptional regulator Nrd R, oligopeptide ABC transporter protein, phosphoribosylamine-glycine ligase, phospho-glucosyltransferase, RplT 50S ribosomal protein L20 and Zinc ABC transporter in strain DPC5607 only. A SNP present in the gene encoding an oligopeptide ABC transporter protein was only present in ISL2+/- strains. SNPs were present in all three strains, these included an ABC

transporter, putative beta lactamase, phosphoribosylaminoimidazole carboxylase ATPase subunit, cardiolipin synthase, tRNA (uracil-5-)-methyltransferase and phosphoglycerate kinase (Table 1). The SNPs detected within protein-coding regions were nonsynonymous SNPs (nsSNP), i.e. they were responsible for amino acid replacements, except in one instance where we detected the presence of a nonsense SNP (position: 174405 in DPC5607) within a phospho-glucosyltransferase gene that resulted in a change from a tyrosine to a stop codon in DPC5607.

3.3.2 SNPs in other locations throughout the genome

Within hypothetical proteins, there were 2 SNPs detected in all three strains (position: 1350384 and 1273462) and eight present in DPC5607 only (Table 1). All of these were nsSNPs. We also detected a number of SNPs within mobile elements i.e. DNA transposases and insertion sequence (IS) elements (Table 1). These included two premature stop codons, resulting in a tryptophan (position: 591976 in DPC5607) and a leucine (position: 1120432 in ISL2+ and DPC5607, respectively), being changed to a stop codon. All other SNPs within the mobile elements were nsSNPs.

Many SNPs were located within intergenic regions (iSNPs). As these are located between coding sequences they can potentially alter the levels of expression of nearby genes. Here we identified a total of 36 iSNPs across the three strains, and within 12 distinct intergenic regions (Table 2). The function of the nearby genes is also described in Table 2. Within these we detected 22 SNPs common to the three strains. There were 9 SNPs unique to DPC5607 and 1 to ISL2- (position: 876628). ISL2+/- had one SNP present in both strains (position: 1140913). ISL2- and DPC5607 also had a common SNP (position: 876563). A number of these SNPs were located near mobile elements, including transposase and IS elements, and hypothetical proteins. We determined the presence of 12 SNPs before a 30S ribosomal protein S15, 10 which were common to the three strain, one was only detected in ISL2- and another was present in ISL2- and DPC5607. SNPs were also detected near a restriction-modification system, position: 1136053 of DPC5607 and position: 1140913 of ISL2+/-. We also detected two SNPs in the three strains (positions: 1513257 and 1513302), near a phosphoribosylaminoimidazole

carboxylase ATPase subunit. One of which resulted in the occurrence of a stop codon sequence, position 1513302.

We also detected nsSNPs in pseudo genes (Table 3). These are dysfunctional relatives of genes that have lost their protein-coding ability or are otherwise no longer expressed in the cell (Balakirev and Ayala, 2003). The predicted original function of these genes, as determined by comparisons with close relatives (Callanan et al., 2008), are described in Table 3. As these genes are non-functioning, it is expected that these SNPs will not have impacted on the phenotypic differences being observed.

4. Discussion

L. helveticus DPC4571 is a Swiss cheese isolate that has been extensively investigated as a starter and adjunct culture in cheese manufacture. It possesses a number of traits that are highly desirable from a cheese production perspective (Slattery et al., 2010). Here we examined two derivatives of DPC4571, which vary with respect to the number of IS elements they possess. IS elements contribute significantly to the genetic variability of prokaryotic organisms (Mira et al., 2002), they are the simplest form of mobile genetic structures capable of causing chromosomal rearrangements and affecting genome plasticity. ISL2 belongs to the IS*5* family (Kaleta et al., 2010). We also assessed *L. helveticus* DPC5607. Pulse field gel electrophoresis (PFGE) profile of this strain presented a very similar pattern to that for DPC4571, suggesting they are very closely related (Kaleta et al., 2009). These three strains exhibited phenotypes that differed from *L. helveticus* DPC4571. The API 50 biochemical assessment revealed that the strains utilise different carbohydrates. Interestingly ISL2+ and ISL2- utilised the same substrates, yet exhibited different growth rates in the different media. To try to better understand why these growth differences were observed, we carried out genome sequencing on the three test strains to identify the presence of SNPs. Here we applied Illumina sequencing technology, which provides the sample throughput level and high-quality data required for accurate SNP discovery and structural variation analysis studies (Mullen et al., 2012). By aligning the sequence reads to DPC4571 genome sequence (Callanan et al., 2008), we able to determine the presence of a number of SNPs.

We did not detect SNPs, nor were any indels (insertions or deletions) observed which may account for the differences observed in the ISL2 content. Genome sequencing did reveal the presence of many nsSNPs in these derivatives, as well as in the genome of DPC5607, which may be the cause of the different growth abilities observed. Within the ISL2+/- derivatives there was only one unique SNP. This was a nsSNP present in an oligopeptide ABC transporter protein. Oligopeptides are an important source of nutrients, but can also serve as signals for intracellular communication. This transport

system plays a role both in the transport of oligopeptides into the cell and in the communication process (Alloing et al., 1994). In lactic acid bacteria, mutations in the oligopeptide transport system have demonstrated an inability to effectively utilise β-casein-derived amino acids. This system is an important step in the proteolytic pathway (Kunji et al., 1995) and the presence of this SNP may be associated with the growth differences of these derivatives compared to DPC4571. A number of SNPs detected, common to the two derivatives and DPC5607, may also be associated with the phenotypic differences observed. These include changes in genes encoding an ABC transporter and a tRNA (uracil-5-)-methyltransferase, as well as to a cardiolipin synthase and purine biosynthesis subunit. These genes are involved in cellular function and maintenance, mutation of which has been associated with alterations in growth efficiency previously (Serricchio and Bütikofer, 2012, Carvalho et al., 2013, Donovan et al., 2001). Notably, we also detected the presence of two SNPs in the intergenic region of ISL2+/- and DPC5607 located near a phosphoribosylaminoimidazole subunit, one of which (position: 1513302) resulted in a premature stop codon sequence, which may be associated with differences observed in the ability of these strains to grow in milk. SNPs were present in the phosphoribosylaminoimidazole subunits which are involved in purine biosynthesis. Deficiencies in purine biosynthesis have been associated with slow milk coagulation of *L. helveticus* strains (Hebert et al., 2001). It would be interesting to assess further if the SNPs of these genes are contributing to growth differences. Finally, we also detected the presence of a common SNP within a phosphoglycerate kinase. This is an enzyme involved in the glycolysis pathway, a principal pathway for the formation of flavour compounds in cheese (McSweeney and Sousa, 2000). Further attention is required to determine if the presence of this SNP affects the phenotypic differences observed.

Nine SNPs, within genes of known function, were unique to DPC5607. These again included genes involved in the transport of cellular components and purine biosythnesis but also in cell division, DNA unwinding, and ribosomal protein binding. As discussed above, mutations present in these genes could contribute to the DPC5607 phenotype (Chédin and Kowalczykowski, 2002, Jankovic et al., 2003, Stelzl et al., 2001). One notable SNP in DPC5607 was

at position 1774405 and resulted in the occurrence of a premature stop codon. This was present in a phospho-glucosyltransferase, a sugar transferase involved in converting glucose to glycogen. This transferase has been involved in initiating biosynthesis of exopolysaccharides (EPS) (Lamothe et al., 2002). While the presence of genes predicted to be involved in EPS synthesis have previously been noted in DPC4571 (Callanan et al., 2008), this strain is not known for its EPS production. However, EPS production has been observed in a number of *L. helveticus* strains including 766 (Robijn et al., 1995), Lb161 (Staaf et al., 2000) and ATCC 15807 (Torino et al., 2001). It has also been observed in *Lactobacillus johnsonii* F19785 (Horn et al., 2013) that mutations in glucosyltransferase can affect surface characteristics and cell aggregation. Ultimately, the effect of this termination requires further investigation.

The remaining SNPs detected were present in hypothetical proteins. As we do not know the function of these genes, we cannot determine if these SNPs are influencing the phenotypic differences observed. Similarly, we detected a number of SNPs within mobile elements but it is not known if these SNPs affect activity. The SNPs within intergenic regions may impact on the expression of nearby genes. Interestingly, a series of SNPs occurred prior to the 30S ribosomal protein S15, which may affect expression in the gene. Ribosomal proteins play an important role in cellular processes, the presence of mutations in these proteins can affect stability of ribosomes, it has been observed that growth rate can be affected by mutants in ribosomal proteins (Stelzl et al., 2001). Further investigation is required to determine if this volume of SNPs near the 30S ribosomal protein affect expression of this gene. SNPs were also detected near a restriction-modification system, these systems protect against foreign DNA, and in *L. helveticus* can play a role in protection against phage attack (Callanan et al., 2008). While we detected the presence of SNPs within pseudo genes, it has been suggested previously that these genes are more prone to SNP occurrence as they are not under selective pressure to maintain functional integrity (Balasubramanian et al., 2002). The occurrence of SNPs in these genes is less likely to impact on the phenotype of the corresponding strains.

5. Conclusion

In conclusion, whole genome comparisons revealed the presence of a number of SNPs in ISL2+, ISL2- and DPC5607 relative to dairy isolate DPC4571. It is likely that, of these, those in genes which are associated with structure, division and cellular processes, as well as proteins involved in transport are of greatest significance. Indeed SNPs in sugar transport genes, oligopeptide transport and purine biosynthesis may explain observed growth differences between the strains. It is anticipated that further analysis will more specifically reveal the impact of these SNPs on protein functionality and strain phenotypes.

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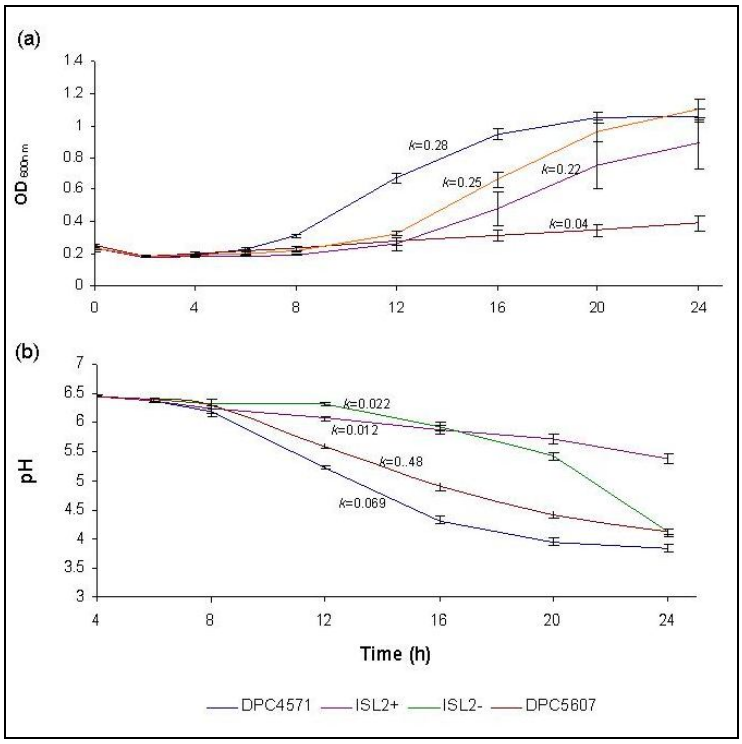

Figure 16: Growth and acidification profiles of *L. helveticus* strains. (a) Growth of strains in mMRS and (b) acidification of 10% RSM over 24 h.
k= growth rate.

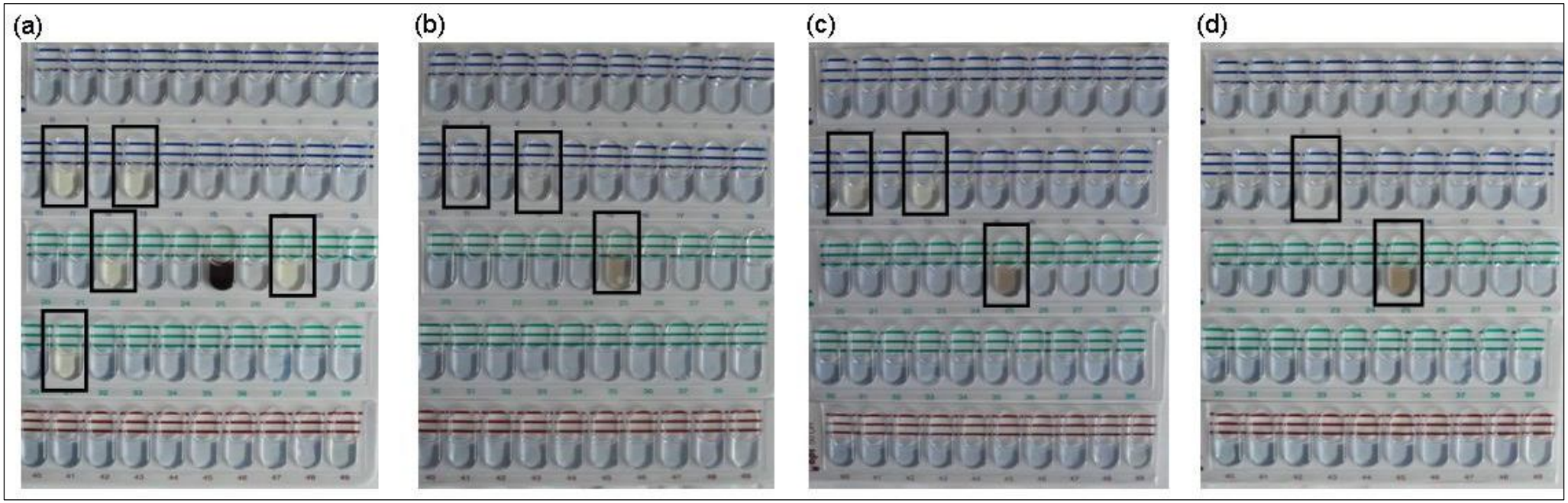

Figure 17: Analysis of carbohydrate fermentation patterns by API biochemical testing.

Positive results are indicated in the highlighted box. The positive well numbers represent the following 11–glucose; 13–mannose; 22–N-acetylglucosame; 25–esculin ferric citrate; 27–cellobiose; 31–saccharose.

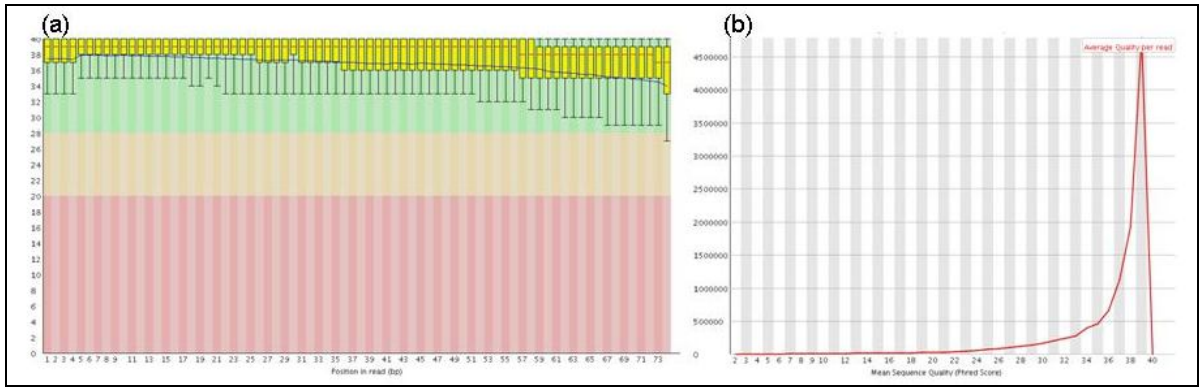

Figure 18: FastQC output from quality check for Illumina raw DNA sequence data.
(a) Represents quality score across all bases, where a score above 20 indicates sufficient quality. (b) Represents quality score distribution over all sequences, the presence of a clean peak indicates tight distribution of sequences with high quality.
Note: While these images were produced for quality checks for each paired-end sequence pool, this image represents the quality check one paired-end file for ISL2+ only. However, this pattern was consistent across all sequence pools (data not shown).

Table 11: SNP detection and location in ISL2+. ISL2- and DPC5607 compared to reference bacterium *Lactobacillus helveticus* DPC 4571.

Chromosome Position					Reference	
ISL2+	**ISL2-**	**5607**	**Gene Name**	**Gene Function**	**AA**	**SNP AA**
			Protein Coding Region			
-	-	945639	lhv_0961	ABC tranporter ATP binding protein	Gly	Ala$^{\perp}$
-	-	1252796	lhv_1272	ATP dependent DNA helicase DinG	Ser	Leu
-	-	844860	lhv_0859	Cell division protein FtsZ	Ala	Val
-	-	1582597	lhv_1614	Transcriptional regulator Nrd R	Ala	Val
-	-	1376908	lhv_1391	Oligopeptide ABC transporter protein	*	*
-	-	1598220	lhv_1625	Phosphoribosylamine-glycine ligase	Thr	Ser$^{\perp}$
-	-	1774405	lhv_1803	Phospho-glucosyltransferase	Tyr	*$^{\blacktriangle}$
-	-	1568653	lhv_1596	rplT 50S ribosomal protein L20	Arg	His
-	-	1809448	lhv_1841	Zinc ABC transporter	Thr	Lys
1927836	1927836	1927836	lhv_1954	ABC transporter	Asp	Val
1711778	1711778	1711778	lhv_1743	Putative beta lactamase	Ser	Phe
1513397	1513397	1513397	lhv_1533	Phosphoribosylaminoimidazole carboxylase ATPase subunit	*	Leu
1376158	1376158	-	lhv_2932	Oligopeptide ABC transporter protein	Thr	Met
1308145	1308145	1308145	lhv_0129	Cardiolipin synthase	Ser	Tyr
1055307	1055307	1055307	lhv_1075	tRNA (uracil-5-)-methyltransferase	Lys	Arg
1055268	1055268	1055268	lhv_1075	tRNA (uracil-5-)-methyltransferase	Pro	Gln
729955	729955	729955	lhv_0744	Phosphoglycerate kinase	Gly	Glu
			SNPs within Hypothetical Proteins			
-	-	388043	lhv_0398	-	Pro	Arg
-	-	422830	lhv_0437	-	Val	Gly
-	-	440846	lhv_0455	-	Gly	Asx
-	-	1096317	lhv_1113	-	Lys	Ile
-	-	1592136	lhv_1621	-	Gly	Glu

Table 1 continued:

Chromosome Position			Gene Name	Gene Function	Reference AA	SNP AA
ISL2+	**ISL2-**	**5607**				
-	-	1796833	lhv_1828	-	His	Leu
1350384	1350384	1350384	lhv_1363	-	Val	Ala
1273462	1273462	1273462	lhv_1288	-	Asn	Ile
			SNPs within Mobile Elements			
-	-	1866362	lhv_1897	Transposase	Arg	Pro
1072787	1072787	1072787	lhv_1092	Putative transposase/ISLhe1	Ile	*▲
-	-	591974	-	ISLhe63	Asn	Ser
-	-	591976	-	ISLhe63	Try	*▲
-	-	591978	-	ISLhe63	Glu	Gly
-	-	591993	-	ISLhe63	Tyr	Cys
-	-	604496	-	ISL2	Ser	Leu
-	-	724972	-	ISLhe63	Ala	Val
-	1859206	1859206	-	ISLhe65	Asn	Thr
1120459	1120459	1120459	-	IS1204	Asn	Ser
1120432	-	1120432	-	IS1203	Leu	*▲
1120276	1120276	1120276	-	IS1202	Pro	His
1120273	-	1120273	-	IS1201	Gly	Val
773847	773847	-	-	ISLhe65	*	*

* asterisk indicates presence of a stop codon
⊥ change to an amino acid with same properties
▲ nonsense SNP

Table 2: Detection of SNPs in intergenic regions and the nearby genes.

Chromosome Position			Reference Base	SNP Base	Gene Location	Gene Name
ISL2+	ISL2-	5607				
-	-	558136	G	A	Transposase	lhv_0570
776057	776057	776057	C	A	Hypothetical protein	lhv_0794
776088	776088	776088	G	T	Hypothetical protein	lhv_0794
-	876628	-	G	C	30S ribosomal protein S15	lhv_0892
876504	876504	876504	A	G	30S ribosomal protein S15	lhv_0892
876510	876510	876510	T	C	30S ribosomal protein S15	lhv_0892
876514	876514	876514	T	C	30S ribosomal protein S15	lhv_0892
876538	876538	876538	T	C	30S ribosomal protein S15	lhv_0892
876541	876541	876541	T	C	30S ribosomal protein S15	lhv_0892
-	876563	876563	G	C	30S ribosomal protein S15	lhv_0892
876577	876577	876577	T	C	30S ribosomal protein S15	lhv_0892
876590	876590	876590	T	C	30S ribosomal protein S15	lhv_0892
876594	876594	876594	C	A	30S ribosomal protein S15	lhv_0892
876596	876596	876596	C	T	30S ribosomal protein S15	lhv_0892
876599	876599	876599	T	C	30S ribosomal protein S15	lhv_0892
-	-	1136053	C	G	Type I restriction-modification system modification subunit	lhv_1154
1140913	1140913	-	A	T	Type I restriction-modification system restriction subunit	lhv_1158
1198759	1198759	1198759	A	C	Hypothetical protein	lhv_1218
1198760	1198760	1198760	G	A	Hypothetical protein	lhv_1218
1215977	1215977	1215977	G	T	Mobile element	-
1216066	1216066	1216066	G	A	Mobile element	-
-	-	1220444	T	C	Transposase IS1201	lhv_1245
-	-	1221425	C	T	Transposase IS1202	lhv_1245

Table 12 continued:

Chromosome Position			Reference Base	SNP Base	Gene Location	Gene Name
ISL2+	ISL2-	5607				
1513257	1513257	1513257	C	A	Phosphoribosylaminoimidazole carboxylase ATPase subunit	lhv_1533
1513302	1513302	1513302	T	A	Phosphoribosylaminoimidazole carboxylase ATPase subunit	lhv_1533
1513307	1513307	1513307	C	A	Phosphoribosylaminoimidazole carboxylase ATPase subunit	lhv_1533
1540300	1540300	1540300	A	C	Mobile element	-
-	-	1587946	A	G	Hypothetical protein	lhv_1618
-	-	1587947	T	C	Hypothetical protein	lhv_1618
-	-	1588580	A	G	Hypothetical protein	lhv_1618
-	-	1588581	A	T	Hypothetical protein	lhv_1618
1817329	1817329	1817329	A	T	Hypothetical protein	lhv_1850
2001447	2001447	2001447	A	T	Amino acid permease	lhv_2036
-	-	2066086	T	A	Hypothetical protein	lhv_2094

Table 13: Occurrence of SNPs in pseudo genes.

Chromosome Position			Predicted Function^
ISL2+	ISL2-	5607	
-	-	38568	Cytosol non-specific dipeptidase
-	-	119152	Phenolic acid decarboxylase
275333	275333	275333	Serine hydroxymethtransferase
509651	509651	509651	Hypothetical protein
-	-	767163	Hypothetical protein
776014	776014	776014	Alkaline phosphatase family
-	-	907859	Phospho beta galactosidase
1549632	1549632	1549632	N-acetylglucosamine kinase
1615570	1615570	1615570	PrtP proteinasr
-	-	1670274	Phenolic acid decarboxylase

^determined by Callanan et al., 2008 supplementary Table S1.

Thesis Discussion

This thesis revealed the benefits of utilising next-generation sequencing to study the overall microbial content of, as well as specific microbes associated with, dairy foods. The field of food microbiology has benefited from the advances in molecular biology and continued to adopt novel strategies to detect, identify and monitor microbes present in food. These technologies emerged to overcome the limitations of classical culture-based approaches, which are biased by an inability to detect microbes present in low numbers or which may be difficult to grow. Such microbial populations deserve attention as they can be of importance with respect to the quality, spoilage or fermentation of foods. The study of the microbial diversity of foods has now evolved to benefit from high-throughput sequencing approaches, after direct nucleic acid extraction from the matrix to be studied.

Nucleic acid extraction is an important step in advance of high-throughput sequencing-based analyses. A high yield of pure DNA that is representative of all of the species occurring in the environment is desirable. However, not all microbial species are equally sensitivity to lytic agents and extraction procedures, which in turn impacts on the success with which nucleic acids are extracted (Randazzo et al., 2009). The more complex the matrix, the more difficult it is to achieve an efficient extraction and to remove impurities that can negatively impact on downstream processes (Pirondini et al., 2010). In the case of dairy-based foods, the presence of natural constituents such as lipids, proteins, carbohydrates and salts (Wilson, 1997) may render extraction difficult and some of these components may act as PCR inhibitors. It is thus very important to choose a suitable DNA extraction method. We determined that the PowerFood^{TM} Microbial DNA Isolation kit (MoBio Laboratories Inc., USA) had the ability to extract metagenomic DNA of high yield and purity, as well as that from specific Gram-positive (*Listeria monocytogenes* EGDe) and Gram-negative (*Salmonella enterica* serovar Typhimurium LT2) pathogens of concern to the dairy industry. It was established that this method was suitable for extracting DNA for subsequent PCR and qPCR applications, even in instances when the target cells were present in low

numbers. This method was employed to extract DNA from dairy environments for all studies described throughout this thesis.

High-throughout sequencing technology allowed us to assess a large number of samples in a single run. We thus assessed 62 Irish Farmhouse cheeses, 47 milk samples and 48 cheeses with or without pink discolouration, respectively, across three separate runs on a Roche 454 GS-FLX sequencer. By targeting the 16S rRNA gene with degenerate primers, we ensured that the vast majority of bacteria (94.6%) could be amplified (Claesson et al., 2010). The analysis of this DNA sequence data requires the need for bioinformatic analysis. Throughout these studies programmes such as BLAST (Altschul et al., 1990), MEGAN (Huson et al., 2007), MOTHUR (Schloss et al., 2009) and QIIME (Caporaso et al., 2010) were utilised, which allowed assignment of sequence reads and identification of the bacterial component of milk and cheeses.

High-throughput sequencing allowed us to detect the presence of a number of bacteria not previously associated with cheese or milk. These included a number of bacteria present at low levels, or which are regarded as being difficult to culture, highlighting the major benefit of utilising this technology. Here we identified for the first time the presence of *Faecalibacterium*, a strict anaerobe typically associated with the gut, which has potential anti-inflammatory effects (Sokol et al., 2009). Notably, this bacterium was only detected in cow's milk cheese, including both raw and pasteurised milk cheeses. We also detected reads corresponding to this bacterium at low levels in Irish cow's milk, pre- and post-pasteurisation, suggesting that this bacterium is present in cheese as a consequence of the contamination of milk. We also detected the presence of *Prevotella* during our studies. *Prevotella* are commensal bacteria in rumen and hind gut of cow and sheep, where they help breakdown proteins and carbohydrates (Yildirim et al., 2010). It is also associated with periodontal disease in humans (Maeda et al., 1998). We detected *Prevotella* in cheeses manufactured from cow and goat's milk (both raw and pasteurised) and at low levels in raw and pasteurised Irish milk, albeit at a reduced level post-pasteurisation. Also, detected for the

first time in Irish milks was *Bacteroides* and *Parabacteroides*, the latter being a reclassification of species previously assigned as *Bacteroides* (Sakamoto and Benno, 2006). The role of *Bacteroides* in the gut is controversial, with some studies suggesting this bacterium contributes to allergies during infancy (Suzuki et al., 2008), while others have shown, in mice trials, that it can protect against cow's milk allergies (Rodriguez et al., 2012). These theories require further investigation. Other microbes that were unexpectedly detected in Irish cheese and milks were two gut associated microbes, *Helcococcus* and *Catenibacterium*. It is not clear what effect, if any, these have on health or quality/safety of dairy products. Other studies employing high-throughput sequencing to investigate the microbiology of dairy products have also revealed a number of unexpected findings, including the presence of *Bifidobacteriaceae* in Oscypek, a sheep milk cheese (Alegria et al., 2012). Sequencing also detected a number of bacteria, not identified by culturing or denaturing gradient gel electrophoresis (DGGE), in Danish raw milk cheeses including *Micrococcus, Pediococcus, Pseudomonas, Halomonas* and *Staphylococcus* (Masoud et al., 2011).

A number of other interesting results were revealed through the use of high-throughput sequencing to study these dairy foods. It was apparent that Irish artisanal cheeses manufactured from unpasteurised, relative to those made from pasteurised, milk had a more diverse bacterial content. We also noted that differences in production could affect microbial composition dramatically e.g. a higher salt content was associated with the absence of *Leuconostoc* and *Pseudomonas*. Differences in the degree of ripening also influenced the microbial content i.e. higher proportions of *Lactobacillus* are associated with hard cheeses compared to soft cheeses. Also, the inclusion of herbs and spices were associated with reduced proportions of lactococci and increases in lactobacilli. When examining the rind of cheese, we observed that the microbial populations present differed considerably from that in the corresponding cheese curd. Indeed, even in instances where the same genera were detected, the proportions present differed greatly.

When comparing raw to pasteurised cow's milk, flow cytometry revealed anticipated differences in the microbial distribution between live and dead bacteria, as reported previously (Gunasekera et al., 2002). Sequencing data, which related to DNA sourced from live cells only, due to the inclusion of nucleic acid stain ethidium monoazide (Rudi et al., 2005), also revealed a greater diversity in the microbiology of pasteurised milk than previous culture based studies have suggested. Of particular note was the detection of the spoilage bacterium *Pseudomonas*, which is thought to be eliminated by pasteurisation. Culturing of the sample suggested that the bacterium was not present, qPCR analysis was consistent with DNA sequencing data in indicating that Pseudomonas survived to some, albeit greatly reduced, extent. It would thus seem that these and other thermo sensitive bacteria are likely in a highly stressed, or viable but non-culturable (VBNC), state.

With respect to food microbiology studies, high-throughput sequencing-based studies have tended to be of more academic than commercial relevance. However, in chapter IV, we highlight the benefits of using this technology to address issues of major concern to the food industry. Here, high-throughput DNA sequencing of 16S rRNA amplicons was performed to profile the bacterial content of cheeses displaying a pink discolouration defect and of controls without discolouration. Three types of cheeses with which this problem has been associated, i.e. Swiss-type cheese, "thermophillic"-Cheddar type cheese and Cheddar cheese with annatto dye, were assessed. Previous microbiological analysis of these affected cheeses has been on the basis of culture-dependent methods only and a microbial cause for this phenomenon was not determined (Martley and Michel, 2001, Park et al., 1967, Shannon and Olson, 1969). The classical approaches taken were not suited to the detection of *Thermus*, an extreme thermophile with unusual growth requirements. The detection of this bacterium highlights the benefit of using culture independent technologies. Our results led to the development of PCR-based methods, including qPCR, for rapid detection and identification of the bacterium. Subsequent cheese trials, spiked with *T. thermophilus*, reproduced a pinking discolouration where this

bacterium was present indicating a strong association with its presence and pink formation. We were also able to develop a rapid screen method for environmental samples from dairy processing plants, where we revealed hot water as a main entry point for this bacterium. Notably, *T. thermophilus* has previously been associated with this environment (Pask-Hughes and Williams, 1975).

While much of the work carried out in this thesis has been based on the use of Roche GS-FLX-based high-throughput sequencing to determine the bacterial content of milk and chesses, another next-generation sequencing technology, which has been widely used due to its ability to produce even larger numbers of DNA sequence reads, albeit originally of shorter length, is the Illumina platform. We utilised this technology to sequence the genomes of three isolates related to the dairy bacterium *L. helveticus* DPC4571. These strains included two derivatives of DPC4571, which differ with respect to the number of IS elements present, i.e. one contained 18 ISL2 elements (ISL2+) while the other had 15 ISL2 elements (ISL2-), in contrast with the 17 ISL2 elements in DPC4571. The third strain investigated was *L. helveticus* DPC5607, a close relative of DPC4571 (Callanan et al., 2008). These three strains exhibited phenotypic differences relative to *L. helveticus* DPC4571, including differences with respect to the extent of, or rapidity with, which they grew in the complex medium mMRS and the milk-based medium, 10% RSM. They also utilised different carbohydrates based on API 50 biochemical assays. We aligned the Illumina sequencing data to that of DPC4571 with a view to identifying genotypic differences that may explain these different growth patterns. Genome sequencing did reveal the presence of many SNPs in these derivatives, as well as in the genome of DPC5607, which may contribute to these phenotypes. These SNPs were frequently associated with structure, division and cellular processes, as well as proteins involved in transport. The ISL2+/- derivatives shared one unique SNP. This was a nonsynonymous SNP (nsSNP) present in an oligopeptide ABC transporter protein. This transport system plays a role both in the transport of oligopeptides into the cell and in the communication process (Alloing et al., 1994). This

system is an important component of the proteolytic pathway and mutations in the oligopeptide transport system have previously resulted in an inability to effectively utilise β-casein-derived amino acids (Kunji et al., 1995) and thus the presence of this SNP may contribute to the growth differences between these derivatives and DPC4571. One notable SNP in DPC5607 was at position 1774405 and resulted in a premature stop codon in the phospho-glucosyltransferase gene i.e. a sugar transferase involved in converting glucose to glycogen (Werning et al., 2012). A number of other SNPs detected in the three genomes sequenced, which may explain the phenotypic differences observed, include those in genes of ABC transporter systems, a tRNA (uracil-5-)-methyltransferase, as well as genes involved in cellular development and DNA formation, such as a cardiolipin synthase, DNA helicase and a purine biosynthesis subunit. These genes are involved in cellular function and maintenance, mutation of which has been associated with alterations in growth efficiency previously (Serricchio and Bütikofer, 2012, Carvalho et al., 2013, Donovan et al., 2001). Notably, deficiencies in purine biosynthesis have been associated with slow milk coagulation of *L. helveticus* strains in the past (Hebert et al., 2001). It would be interesting to carry out further investigations to determine if the SNPs in these genes are contributing to growth differences. We also detected the presence of a common SNP within a phosphoglycerate kinase, an enzyme involved in the glycolysis pathway, a principal pathway for the formation of flavour compounds in cheese (McSweeney and Sousa, 2000). Ultimately, the application of whole genome comparisons has been of value by revealing the presence of SNPs in ISL2+, ISL2- and DPC5607 which may explain the observed growth differences between the strains.

In conclusion, it is apparent that high-throughput DNA sequencing platforms are powerful tools that can be applied to study the microbiota of dairy foods. The application of these technologies has allowed us to determine the presence of a number of bacteria which have not been detected by traditional culturing methods; it has allowed the identification of a bacterium, *T. thermophilus*, which is associated with the formation of a pink defect in cheese, a problem which has remained unsolved for

many years. These platforms have also demonstrated the value of genome sequencing when comparing closely related strains. It is anticipated that high-throughput DNA sequencing will continue to provide new insights for food microbiologists for quite some time to come.

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